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(54) Title: *CRYPTOSPORIDIUM* POLYPEPTIDES, NUCLEIC ACID, VECTORS AND METHODS OF USE

(57) Abstract

A biologically pure polypeptide comprises a biologically pure, isolated peptides capable of specifically binding to a distinct anti-*Cryptosporidium* antibody. The polypeptides bind to different subgroups of anti-*Cryptosporidium* antibodies that selectively bind the > 900 kD, the 68 kD and 95 kD, the 45 kD, the 23 kD, or the 15 kD and 32 to 35 kD app. MW polypeptides from *C. parvum* among other *Cryptosporidium* polypeptides. Biologically pure DNA and RNA segments encode the polypeptides of this invention. Fusion proteins comprise at least one of the polypeptides and a second unrelated polypeptide operably coupled to the polypeptide of the invention. Fusion polydeoxyribonucleotides or deoxypolynucleotides comprise the RNA or DNA of the invention and a second unrelated deoxyribopolynucleotide or deoxypolynucleotide. A hybrid vector capable of replication, transcription and expression of DNA segments operably coupled thereto carries a DNA sequence expressing the polypeptide of this invention or a fusion protein thereof. Hybrid eukaryotic and prokaryotic hosts carry the hybrid vector of the invention. The polypeptides or anti-*Cryptosporidium* antibodies are used to retard, inhibit or counter *Cryptosporidium* infection and/or development in a subject. Infection diagnostic methods employ at least one of the polypeptides or anti-*Cryptosporidium* antibodies. Diagnostic kits comprise at least one of the polypeptides or anti-*Cryptosporidium* antibodies, and instructions for their use.

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Cryptosporidium Polypeptides, Nucleic Acid, Vectors. and Methods of Use.

BACKGROUND OF THE INVENTION

This invention was developed at least partially with U.S. Government support under National Institutes of Health Grant Nos. AI-29882 and R43 AI 30295-01A1. The U.S. Government may have certain rights in this invention.

Field of the Invention

This invention relates to a novel polypeptide comprising an amino acid sequence capable of specifically binding antibodies raised against the protozoan pathogen *Cryptosporidium spp. (Cryptosporidium)*. This invention also relates to DNA and RNA segments which encode such polypeptides. These polypeptides bind antibodies that afford protection against, ameliorate symptoms of, and accelerate recovery from, infection by this pathogen. These polypeptides may be produced from DNA or RNA sequences encoding them, and they may be used for the immunization of humans or animals against infection by *Cryptosporidium*. These polypeptides may also be used for the production of antibodies suitable to counter *Cryptosporidium* infection. Finally, this invention relates to the use of these polypeptides and antibodies raised against them in diagnosing infection by *Cryptosporidium* and diagnostic kits.

Description of the Background

Cryptosporidium are parasitic agents causing infection in a wide variety of animals including birds, reptiles and mammals. *C. parvum* is believed to be the major cause of disease in humans and domestic animals. The disease is acute and self-limited in immunocompetent humans. It, however, causes a more severe and potentially lethal disease in persons affected with AIDS. In spite of the morbidity of cryptosporidiosis in AIDS patients, no

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effective immunotherapy or chemotherapy is available at the present time to counter this disease. Moreover, the understanding of the biology and biochemistry of *Cryptosporidium*, as well as the pathophysiology of cryptosporidiosis is still at an early stage.

C. parvum has been classified based on its ultrastructural features as a member of the phylum *Apicomplexa*. "Zoites" such as sporozoites, merozoites, and tachyzoites are the invasive stages of the *Apicomplexa*. The zoites are extracellular and have a unique trilaminar membrane, called the pellicle, which appears to mediate the attachment of the parasite to the host cell membrane. The pellicle is associated with subpellicular structures that are involved in motility. The zoites also share an anterior apical complex composed of specialized secretory organelles (rhoptries, micronemes and dense granules). These organelles secrete products which appear to facilitate the entry of the zoite into the host cell and the generation of the parasitophorous vacuole.

The *C. parvum* infection is initiated by the ingestion of oocysts, the excystation of oocysts with release of sporozoites and the invasion of gut epithelial cells by sporozoites. Thereafter, the intracellular forms mature and release new daughter merozoites which reinvade the gut epithelial cells. *C. parvum* also has a sexual cycle. The sexual cycle of *C. parvum* also occurs in the gut and results in the production of sporulated oocysts, some of which may excyst before being shed. In persistent infection of an immunocompromised host, both the merozoite and the endogenously produced sporozoite may contribute to the ongoing invasion by *C. parvum*. The relative contribution of each stage, however, remains unclear.

Sporozoites and merozoites are the only stages of *C. parvum* which are free in the gut and, therefore, accessible to neutralization by luminal antibody. In other Apicomplexan zoites, e.g., *Plasmodium*, *Eimeria*, and *Toxoplasma*, the pellicle and apicle complex proteins are targets of invasion-inhibiting antibodies *in vitro*, and

neutralization antibodies *in vivo*. Polypeptides localized in the pellicles and apicle complex of *C. parvum* zoites are likely targets of endogenous host immune responses. To date, the most promising treatment for cryptosporidiosis or infection by *C. parvum*, is the passive oral transfer of anti-*C. parvum* hyperimmune bovine colostral immunoglobulin (HBC Ig). HBC Ig has been shown to react with numerous oocyst and sporozoite proteins on Western blots and to be therapeutic in neonatal mice. Whole hyperimmune bovine colostrum (HBC) has also been reported to inhibit infection by *C. parvum*. Duodenal infusions of HBC have been reported to ameliorate *C. parvum* infection in AIDS or other immuno-compromised patients.

GP15, GP20 and P23 are examples of sporozoite proteins or glycoproteins which are exposed on the surface of *C. parvum*. These antigens are examples of targets of monoclonal antibodies raised against the corresponding oocysts/sporozoites. These monoclonal antibodies have been shown to prevent or attenuate infection in studies using animals challenged with *C. parvum*. Monoclonal antibodies such as 17.41 and 18.44 have been reported to partially protect mice from *C. parvum* infection, although the sizes of the target antigens are still unclear. Monoclonal antibody 18.44 recognizes a non-peptide antigen. In some instances, the epitope recognized by the monoclonal antibodies has been found in both sporozoites and merozoites.

Thus, there is still a need for agents useful for the immunotherapy of cryptosporidiosis in both uncompromised and immunocompromised subjects, e.g. AIDS, patients, which would prevent or limit the disease's manifestations. There is also still a need for an agent useful for the detection of ongoing *C. parvum* invasion, particularly in its early stages.

SUMMARY OF THE INVENTION

This invention relates to a biologically pure polypeptide comprising a biologically pure, isolated

peptide capable of selectively and specifically binding to anti-*Cryptosporidium* antibodies.

This invention also relates to biologically pure DNA and RNA segments that encode the polypeptides described above.

Also part of this invention is a fusion protein comprising one of the polypeptides described above and a second unrelated polypeptide expressed by a regulatory DNA segment operably coupled to the DNA segment described above that encodes the polypeptide of this invention. Still part of this invention are fusion RNA and DNA polymers comprising the RNA or DNA of this invention and a second unrelated polyRNA or polyDNA segment.

In addition, this invention comprises a hybrid vector comprising the DNA segment described above operatively coupled thereto, and a hybrid eukaryotic or prokaryotic host carrying the hybrid vector of this invention.

This invention also relates to a method of retarding, inhibiting, or countering *Cryptosporidium* infection of a subject's cells comprising administering to a subject in need of such treatment an amount of an *anti-Cryptosporidium* antibody effective to retard the invasion by and/or development of *Cryptosporidium* of the subject's cells.

Also provided herein is a method of retarding, inhibiting or countering *C. parvum* infection of a subject's cells comprising administering to a subject in need of such treatment an amount of one of the polypeptide of this invention capable of eliciting from the subject a cell invasion and/or development inhibitory amount of anti-*C. parvum* antibodies.

In addition, this invention comprises a method of diagnosing *Cryptosporidium* infection of a subject, comprising

contacting a body substance obtained from the subject with an anti-*Cryptosporidium* antibody; and

detecting any selective binding of the antibody to any antigenic *Cryptosporidium* peptide present in the body substance.

This invention, in addition to the above, also encompasses a method of diagnosing *Cryptosporidium* infection of a subject, comprising

contacting a body fluid obtained from the subject with the polypeptide of this invention; and

detecting any selective binding of the polypeptide to any anti-*Cryptosporidium* antibodies in the body fluid.

Also part of this invention is a *Cryptosporidium* diagnostic kit, comprising

anti-Cryptosporidium specific antibodies; and

instructions for the use of the kit.

Furthermore, this invention also provides a *Cryptosporidium* infection diagnostic kit, comprising

the polypeptide of this invention; and

instructions for use of the kit.

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily perceived as the same becomes better understood by reference to the following drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the inhibition of *Cryptosporidium* infection of epithelial cells by HBC Ig. 100-1000 μg/ml IgG resulted in a significant reduction ($p<0.01$) in the mean number of intracellular parasites/MDCK (Madin-Darby canine kidney) cell of up to 61% relative to fetal calf serum (FCS) and of up to 55% relative to sham HBC (SHAM-HBC) Ig controls, while no inhibition was observed at lower HBC Ig concentrations (≤50 μg/ml IgG).

Figure 2 depicts the effect of SHAM-HBC Ig on *C. parvum* infection of MDCK cells. No significant inhibition was observed for SHAM-HBC Ig at concentrations ranging from 1500 μg/ml to 75 μg/ml IgG with respect to FCS controls ($p<0.01$).

Figure 3 depicts the results of another specific anti-*Cryptosporidium* antibody assay. HBC Ig antibodies (50-100 μg/ml IgG) eluted from *C. parvum* Western blots were utilized in a MDCK *in vitro* assay. The experimental data

were normalized to SHAM-HBC IgG controls (75 μg/ml IgG). Significant inhibition of *Cryptosporidium* infectivity was observed for cultures treated with anti-*Cryptosporidium* Ab eluted from Western blot (Eluted Ab) and HBC Ig (100 μg/ml IgG) (HBC Ig) with respect to SHAM-HBC Ig (100 μg/ml IgG) (SHAM), glycine buffer and FCS controls ($p<0.01$).

Figure 4 shows a Western Blot of *C. parvum* proteins developed with HBC Ig or SHAM-HBC Ig. Oocyst/sporozoite proteins were either blotted (lane 1) or immunoprecipitated with HBC Ig at dilutions 1/1,000 (lane 2), 1/5,000 (lane 3), and 1/10,000 (lane 4) and with SHAM-HBC Ig at the same dilutions as above (lanes 5-7). After electrophoresis, Western blots were developed with HBC Ig (lanes 1-4) or SHAM-HBC Ig (lanes 5-7) followed by [^{125}I]-Protein G, and autoradiographed.

Figure 5(A)- Surface proteins of *C.parvum* sporozoites recognized by HBC Ig. Radioiodinated sporozoites were disrupted, and an aliquot of the membrane proteins was directly resuspended in SB and electrophoresed (lane 1). Alternatively, radiolabeled sporozoite membranes were SDS solubilized (lane 2) or Triton X-100 solubilized (lane 3) prior to immunoprecipitation with affinity bound HBC Ig. Proteins were resolved by SDS-PAGE, and autoradiographed for 8 hrs.

Figure 5(B)- Detail of autoradiogram of lanes 2 and 3 exposed for 24 hrs. The >900 kD and about 250 kD apparent molecular weight (app. MW) molecules are indicated by arrows.

Figure 6 depicts the in vitro inhibition of *C. parvum* infectivity by antibodies raised against the recombinantly obtained proteins encoded by S19, S34 and S2 *Crytosporidium* clones. Col. 1: anti-S19 ascites (dilution 1/20). Col. 2: anti-S34 ascites (dilution 1/20). Col. 3: anti-S2 ascites (dilution 1/20). Col. 4: HBC Ig (dilution 1/100). Col. 5: anti-GP900 ascites (dilution 1/20). Col. 6: SHAM-HBC Ig (dilution 1/100). Col. 7: FCS control.

Other objects, advantages and features of the present invention will become apparent to those skilled in the art from the following discussion.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention arose from a desire by the inventors to provide novel compositions suitable for therapeutic use in *C. parvum* infection, such as the active and passive immunotherapy of *C. parvum* infected subjects or to ameliorate symptoms produced by infection of the parasite. The polypeptides of this invention are also useful for the early detection of *Cryptosporidium* infection. The inventors targeted the protein components of the parasite for their work. Protein extracts of *C. parvum* were screened for polypeptide components evidencing specific binding to antibodies raised against the parasite. In order to isolate the DNA encoding such polypeptides, viral DNA expression libraries were constructed and then screened with polyclonal anti-*C. parvum* antibodies reactive with sporozoites and oocysts of the parasite. A large number of colonies were screened. The number of clones was significantly reduced when only those clones (57) that positively bound to the specific antibodies were kept. The antibodies binding to the polypeptides produced by the 57 clones were then purified by elution from the lawns containing the colonies, and employed to help to determine the apparent molecular weights (app. MW) of the different polypeptides of the parasite. These purified antibodies were also used to determine the location of endogenous immunofluorescent antigens (IFA) in the intact *C. parvum* organism.

A number of clones were identified and eventually segregated into distinct immunogenic *C. parvum* groups. Five of these distinct groups of polypeptides were then localized by IFA to the sporozoite pellicle and apical complex regions of the *C. parvum* organism, and the apparent molecular weights of the endogenous polypeptides were determined.

The above approach relying on the isolation of *anti-C. parvum* antibodies recognizing the cloned polypeptides afford advantages over a monoclonal antibody (MAb) approach. The antibodies utilized by the inventors are typically polyclonal antibodies that recognize multiple epitopes on the target polypeptide. The monoclonal antibodies elicited by the GP900 protein are monoepitopic, and carbohydrate specific, which renders them inefective for screening a cDNA library. Moreover, in the present method, large amounts of recombinant polypeptide may be produced to, in turn, obtain a corresponding large quantity of polyclonal antibodies or to make the antibodies in animals. Furthermore, once polyclonal eluted antibodies recognizing the antigen of interest have been identified, the corresponding genes, by definition, are in hand for further analysis and use.

This invention provides a biologically pure polypeptide comprising a biologically pure, isolated peptide capable of specifically binding to anti-*C. parvum* antibodies, and optionally a second unrelated polypeptide to form a fusion protein.

The binding of the present peptides may be attained by contacting the peptide for each clone provided herein with polyclonal antibodies raised against *Cryptosporidium* or with monoclonal antibodies obtained by the fusion of, e.g., a myeloma cell line with an anti-*Cryptosporidium* antibody producing lymphocyte. The binding of the peptide in the biologically pure peptide to the antibodies may be determined by detection with anti-immunoglobulin antibodies conjugated to an indicator moiety which can be detected after development with a colorometric reagent, among other methods. For example, nitrocellulose membranes containing the biologically pure polypeptide may be incubated with the antibodies, washed, and then incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Promega) prior to development with, e.g., the colorometric reagents nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. Alternatively, the indicator moiety may be radiolabeled,

e.g., ^{125}I labeled protein A, which binds to the antibodies. The detection in this instance is by exposure to radiographic film (see, e.g., Example 12).

In one preferred embodiment, the peptide in the polypeptide is capable of binding to a subgroup of antibodies that selectively bind the S34 polypeptide from *C. parvum,* polypetpides of clones encoding other peptides encompassed by the >900 kD app. MW protein or fusion proteins from clones that hybridize to the >900 kD app. MW DNA insert. All molecular wirghts reported in this patent were obtained by denaturing electrophoresis with BIS-polyacrylamide gels, unless otherwise specified. This subgroup of antibodies is capable of binding the >900 kD app. MW peptide from *C. parvum,* and they may be obtained by challenging an animal with *C. parvum* as shown in the examples. This subgroup of antibodies may be isolated from a mixture of polyclonal antibodies, as shown in the examples or by elution from the colonies that they bind to, after a polypeptide of a given specificity is produced by recombinant DNA technology, as also shown in the examples. These antibodies also specifically bind the S34 polypeptide, other peptides encompassed by the >900 kD app. MW protein, and fusion proteins thereof. Polypeptides other than the S34 polypeptide may be obtained by screening a *C. parvum* DNA library(ies) with the S34 DNA and monoclonal and polyclonal antibodies.

In a preferred embodiment, the peptide in the polypeptide comprises a glycopeptide of app. MW >900 kD. This glycopeptide corresponds to at least a portion of the endogenous antigen isolated from the *C. parvum* parasite, as shown in the examples. In a still more preferred embodiment of the invention the peptide in the polypeptide comprises the amino acid sequence of the open reading frame (ORF) shown in Table 1 below or other open reading frames of the S34 DNA. Still other preferred polypeptides are those expressed by the S34 clone. In a most preferred embodiment, the polypeptide and fusion protein comprise one or more of the peptides shown in Table 4 below.

Table 1

Amino acid sequence of large S34 ORF

```
 81                                                  91
       gln his phe leu leu gln leu glu pro gln asp asn gln gln leu leu gln leu
101                                                 111
glu val gln ala asn gln leu leu leu pro leu ser lys ala thr thr thr thr thr
121                                                 131
leu asn pro ile ile thr thr thr thr gln lys pro thr thr thr thr thr thr lys val
141                                                 151
pro gly lys pro pro ile ala thr thr thr thr leu lys pro ile val thr thr thr
161                                                 171
thr thr lys ala thr thr thr thr thr thr thr val pro thr thr thr thr thr thr lys
181                                                 191
arg asp glu met thr thr thr thr thr pro leu pro asp ile gly asp ile glu ile thr
201                                                 211
pro ile pro ile glu lys met leu asp lys tyr thr arg met ile tyr asp tyr asn ser
221                                                 231
gly leu leu leu asp ser asn asp glu pro ile pro gly ser gln ala gly gln ile ala
241                                                 251
asp thr ser asn leu phe pro gly ser asn ser gln glu tyr trp phe thr asn
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In another embodiment, the polypeptide of this invention is capable of binding a subgroup of anti-*C. parvum* antibodies that selectively bind a peptide selected from the group consisting of 15 and 32 to 35 kD (doublet) app. MW peptides from *C. parvum*, polypeptides of clones encoding other peptides encompassed by the 15 and 32 to 35 kD protein and fusion proteins of clones that hybridize to the S34 DNA insert. The subgroup of antibodies binding the 15 and 35 kD app. MW bands show substantially no binding affinity for the polypeptides expressed by the other clones but bind with specificity to the S24 protein, to other peptides encompassed by the 15 and 32 to 35 kD protein, and fusion proteins thereof. Polypeptides other than the S24 polypeptide may be obtained by screening a *C. parvum* DNA library(ies) with the S24 DNA and monoclonal and polyclonal antibodies. Still more preferred is a peptide that has an app. MW selected from the group consisting of 15 and 32 to 35 kD. These polypeptides cross-react with the peptide expressed by the S24 clone from *C. parvum*. In a still more preferred embodiment, the polypeptide and fusion protein of the invention comprise a peptide selected from the group peptides of Table 10 below.

In another aspect of this invention, the peptide in the polypeptide is capable of binding to a subgroup of antibodies that selectively bind to a peptide selected from the group consisting of the 68 and 95 kD app. MW peptides from *C. parvum*, polylpeptides of clones encoding other peptides encompassed by the entire protein, and fusion proteins of clones that hybridize to the S19 DNA insert. The subgroup of antibodies binding to this particularly polypeptide of the S19 clone was found to be substantially non-overlapping with the group of antibodies that bind to the peptide of the S34 clone. In another preferred embodiment, the peptide has an app. MW selected from the group consisting of 68 kD and 95 kD. These peptides having an app. MW of 68 kD and 95 kD may be isolated in impure form from a *C. parvum* lysate electrophoresed on a gel, and then specifically bound with antibodies for this specific

clone subgroup. The peptides and polypeptides may be prepared in substantially pure form by cloning as described herein. Also preferred is the peptide expressed by the S19 clone. In addition, other peptides binding to this subgroup of antibodies may also be obtained by recloning and expression of the DNA segments encoding them in an appropriate host as is known in the art. This subgroup of antibodies also binds to the S19 polypeptide, to other peptides encompassed by the 15 and 32 to 35 kD proteins, and fusion proteins thereof. Polypeptides comprising a peptide other than the S19 peptide may be obtained by screening a *C. parvum* DNA library(ies) with the S19 DNA and monoclonal and polyclonal antibodies. In a most preferred embodiment the polypeptide and fusion protein comprise a peptide shown in Table 6 below. Substantially no cross-reactivity exists between this group of peptides and the peptides of the other groups.

In another embodiment, the peptide in the polypeptide is capable of binding to a subgroup of anti-*C. parvum* antibodies that selectively bind to the 45 kD app. MW peptide from *C. parvum*. Also preferred, is the peptide expressed by the S2 clone, polylpeptides of clones encoding other peptides encompassed by the entire protein, and fusion proteins of clones that hybridize to the S2 DNA insert. As in the previous cases, the subgroup of antibodies capable of binding to the 45 kD app. MW peptide from *C. parvum* may be isolated by elution after binding to the specific polypeptide expressed by a recombinant host carrying, e.g., the S2 clone. Monoclonal antibodies may also be obtained by methods known in the art such as the fusion of a lymphocyte expressing an antibody of this specificity with, e.g., a myeloma to form an antibody-producing hybridoma cell line of this given specificity. Substantially no overlapping or cross-reactivity has been found for the 45 kD app. MW peptide from *C.parvum* with the other polypeptides from clones corresponding to a different group. In a preferred embodiment of the polypeptide, the peptide has an app. MW of 45 kD. Such peptide may be

produced by electrophoresing a lysate of *C. parvum*, Western blotting of the protein material and binding of polyclonal antibodies having specificity for the 45 kD peptide band thereto. The subgroup of antibodies selectively binding the 45 kD app. MW polypeptide also bind the S2 group of polypeptides, other peptides encompassed by the 45 kD protein, and fusion proteins thereof. Polypeptides other than the S2 polypeptide may be obtained by screening a *C. parvum* DNA library(ies) with the S2 DNA and monoclonal and polyclonal antibodies.

In another embodiment of the polypeptide, the peptide is capable of binding a subgroup of anti-*C. parvum* antibodies that selectively bind the 23 kD app. MW polypeptide from *C. parvum*, other peptides encompassed by the S7 protein or fusion proteins thereof. Preferred are the polypeptides having a 23 kD app. MW and that expressed by the S7 clone, polypeptide of clones encoding other peptides and fusion proteins of clones that hybridize to the S7 clone. The subgroup of antibodies shown to bind the 23 kD app. MW polypeptide also binds to the polypeptide expressed by the S7 clone group and fusion proteins thereof. This subgroup of antibodies shows substantially no-cross reactivity with other polypeptides from a different group of clones. In a preferred embodiment, the polypeptide and fusion protein comprise one or more peptide(s) shown in Table 8 below. In another preferred embodiment, the peptide in the polypeptide has an apparent molecular weight of 23 kD. Another preferred polypeptide is that expressed by the S7 clone. In still another preferred embodiment, the peptide in the polypeptide comprises fusion protein of the 23 kD polypeptide and a second unrelated peptide. In a still more preferred embodiment, the peptide comprises the peptide having the open frame sequence shown in Table 2 below, or other open reading frame sequences of the S24 DNA. In a most preferred embodiment, the polypeptide or hybrid protein comprise one or more of the peptides shown in Table 8 below.

Table 2

Amino acid sequence of large S7 ORF

```
                                                                  ser ile glu met
ser thr leu val arg lys leu ala pro asn phe thr ala glu ala val met ala asp gly
ser phe lys lys val ser leu ser asp tyr arg gly lys tyr val val leu phe phe tyr
pro leu asn phe thr phe val cys pro ser glu ile leu ala phe asn gln ala gln lys
asp phe glu lys leu gly val gln leu leu ser cys ala gln leu ile leu asn thr pro
met leu his gly asp val leu leu leu asn lys val glu leu asp gln ser ile ser his
leu ser leu thr his leu ile gln leu ala arg thr met val tyr phe leu glu glu glu
gly ile ala leu arg gly leu phe ile ile asp lys glu gly arg val val arg ser glu
val ile tyr asp leu pro leu gly arg ser val glu glu thr leu arg val ile asp ala
leu gln phe thr glu thr tyr gly glu val cys pro ala asn trp lys lys gly gln lys
gly met ser ala thr his glu gly val ser ser tyr leu lys asp ser phe
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Also part of this invention is a composition of matter that comprises

the polypeptide of this invention; and

a carrier, preferably a biologically-acceptable carrier, and more prefrably a pharmaceutically-acceptable carrier.

Typical carriers are aqueous carriers such as water, buffered aqueous solutions, aqueous alcoholic mixtures, and the like. Compositions comprising carriers that are for pharmaceutical use, particularly for use in humans, comprise a carrier that is pharmaceutically-acceptable. Examples of such carriers are known in the art and need therefore not be provided herein.

The composition may comprise about 0.01 to 30 wt% of the polypeptide, and preferably about 2 to 20 wt% thereof. However, other proportions of the carrier to the polypeptide may also be utilized as an artisan will determine by the effectiveness of the treatment. The composition may further comprise other components such as an adjuvant, coloring, a pH adjuster, a filler, and the like, as is known in the art.

The carrier may be a solid or liquid carrier, depending on the route of administration. Typically, the polypeptides of the invention are administrated by the intravenous, subcutaneous or other systemic routes. Given the nature of polypeptides, they are preferably not administrated orally since they are rapidly degraded by the acid pH of the stomach.

Also provided herein is a biologically pure DNA segment encoding the polypeptide of the invention. In addition, provided herein are the DNA sequences for both strands of each clone. Moreover, given the degeneracy of the genetic code, there may be multiple DNA sequences encoding the same polypeptide. All are part of this invention. In a preferred embodiment of the invention, the DNA segment is selected from the group consisting of the DNA fragments of Tables 4, 6, 8, and 10, combinations and repeats thereof, and complementary and degenerate sequences thereof encoding

the polypeptide(s). The DNA segments and fragments of different *Cryptosporidium* clone groups are shown in the tables provided with the examples. Also provided in the tables are the deduced amino acid sequences obtained by reading the two strands of DNA in all three possible reading frames. These amino acid sequences, however, may comprise some portions that are not part of the final polypeptide since they were obtained by translation of genomic polynucleotides and not from cDNA fragments. Thus, when transcription occurs, introns may be excised and the resulting polypeptide may have a substantially different amino acid sequence, be it because of a change in the reading frame or because of a portion was deleted at the RNA level. It is also possible that the deduced amino acid sequences represent solely a portion of the endogenous antigens, although they may comprise more than one epitope. In still another preferred embodiment, the DNA segment of this invention further comprises a second unrelated DNA sequence, such as a regulatory DNA sequence or any generic DNA sequence to add a large peptide to increase the immunogenicity of the polypeptide, that is operably coupled thereto. The addition of a regulatory DNA segment as the unrelated DNA segment permits the increased production of the expressed gene product, i.e., the polypeptide or hybrid protein.

Also part of this invention is an RNA segment that encodes the polypeptide of this invention. The different polypeptides are encoded by different RNA segments. In addition, the RNA segments may encompass degenerate sequences that encode the same polypeptide. All these are part of this invention. Also provided herein are RNA segments comprising substantially pure, isolated, RNA fragments corresponding to both strands of the DNA segments encoding the polypeptides of all five groups, combinations thereof and repeats thereof. A more preferred RNA segment of this invention comprises an RNA fragment selected from the group consisting of polyribonucleotide fragments corresponding to the DNA fragments of Tables 4, 6, 8 and

10, combinations and repreats thereof, and complementary and degenerate sequences thereof encoding the polypeptide of this invention. The RNA segments of this invention may be produced by transcription of the DNA segments disclosed herein by methods known in the art. In another preferred embodiment, the RNA segment further comprises an unrelated RNA segment that is operatively linked to the RNA segment of the invention.

Also provided herein is a fusion protein that comprises the polypeptide of the invention in all its different antigenic forms and a second unrelated polypeptide encoded by, e.g., a DNA segment operably coupled to the DNA segment encoding the polypeptide of the invention. An example of the second unrelated polypeptide is beta-galactosidase, where the DNA segment encoding this gene product also contains regulatory sequences. However, other polypeptides may also be used, such as to provide a large proteic component to increase immunogenicity. If the gene encoding the polypeptide of the invention is cloned within the beta-galactosidase gene, the two polypeptides may be expressed as a fusion protein and the amount of fusion protein produced is controlled by the regulatory sequences of the beta-galactosidase gene.

Also part of this invention is a hybrid vector, that comprises

a vector capable of replication, transcription and expression of DNA segments operably coupled thereto; and

a DNA segment encoding a polypeptide of this invention comprising at least one of the five immunologically different peptide groups disclosed herein operatively coupled thereto, wherein when the vector is placed in an appropriate host it can express the polypeptide encoded by the DNA segment. Examples of such vectors are pGex (Pharmacia), baculovirus, pET-9d (Novagen) or pRSET T7 (Invitrogen). However, other vectors may also be utilized. The vector may be a eukaryotic or a prokaryotic vector depending on the host selected for transfected and in which the gene product is going to be expressed.

This invention also encompasses a hybrid host carrying the hybrid vector of the invention. Examples of hosts suitable for use herein are prokaryotic and eukaryotic hosts such as *E. coli* K12 and related bacteria, and Sf9 or Sf21 insect cells (*Spodoptera frugiperda*), and chinese hamster ovary cells. However, other hosts may also be utilized.

Still part of this invention is another hybrid vector, that comprises

a vector capable of replication, transcription and expression of DNA segments operably coupled thereto; and

a DNA segment comprising a DNA fragment encoding at least one of the polypeptides of the invention and a second unrelated DNA segment, both sequences being operably coupled to one another and to the vector. The preparation of the hybrid vector described above is known in the art and need not be further described herein (Smith, D., and Johnson, K., "Single Step Purification of Polypeptides Expressed in *E. coli* as Fusions with Glutathione S-transferase", Gene 67:31(1988); Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes", Meth. Enzymol. 185:60-89(1990)).

Also an important part of this invention is a method of diagnosing *Cryptosporidium* infection, that comprises

contacting a body substance with an anti-*Cryptosporidium* antibody having specificity for the polypeptide of this invention; and

detecting any selective binding of the antibody to any antigenic *Cryptosporidium* peptides present in the body substance. The detection of the antibody-polypeptide complex may be conducted by any method known in the art. This includes solid phase, double antibody, sandwich double antibody, and triple antibody assays, and the like, including ELISA assays. Also suitable for use herein are enzyme-linked immunoassays and radioactively labeled assays.

Also provided herein is a method of diagnosing *Cryptosporidium* infection, that comprises

contacting a body substance with one of the polypeptides of this invention; and

detecting any selective binding of the polypeptide to any anti-*Cryptosporidium* antibodies in the body substance. As in the previous case, the present antibody-polypeptide binding complex may be detected by a variety of methods such as those listed above. Examples of body substances are stools and other liquid or solid body output or tissue samples obtained from a subject. Examples of body fluids are blood, serum, saliva, urine, and the like. Methods for the preparation of the body substance and the body fluid are standard in the art and need not be further detailed herein (see, for example, Manual of Clinical Microbiology, Chapter 8, "Collection, Handling and Processing of Specimens", 4th edition, Eds, Lennette, E.H., Balows, A., Hausler, W.J. and Shadorny, A.J., American Society for Microbiology (1986)).

The immunotherapy of cryptosporidiosis in humans and animals may be conducted by the oral (intraluminal gastrointestinal) administration of the antibodies of the invention to patients with *cryptosporidiosis* to effectively reduce their symptomatology. In order to reduce to practice the invention, one of the present inventors and others conducted a multiplicity of experiments and made the following observations.

(1) The *in vivo* protective capacity of HBC was correlated with a significant inhibition of *Crysptosporidium* infection of MDCK cells by HBC Ig of the same lot, in a reproducible epithelial cell-*Crysptosporidium* assay. In a newborn calf model of acute *crysptosporidiosis*, HBC was shown to protect the animal from *Crysptosporidium* infection, to reduce the oocysts output to below the limit of detection, and to produce substantially no dehydration in the HBC treated animals. Moreover, an HBC Ig preparation was also shown to be protective in a therapeutic neonatal model of *crysptosporidiosis*, confirming

that immune colostrum effectively and significantly reduces the infectivity and/or proliferation of *Crysptosporidium*. The *in vitro* inhibition of the invasion and intracellular development of *Crysptosporidium* was shown by one of the inventors and others to occur as a function of anti-*Crysptosporidium* titer as evidenced by its correlation with the corresponding immunoglobulin concentration in protective colostrum, and by the lack of biological activity of SHAM colostrum. In a supportive experiment, HBC Ig was also shown to significantly inhibit *C. parvum* infectivity in the Caco-2 cell line.

(2) The *in vitro* ability of HBC to prevent *Crysptosporidium* infectivity was shown to be, in fact, mediated by specific anti-*Crysptosporidium* antibodies and not by other antibodies or components, as shown by the inhibitory effect evidenced by the total antibodies eluted from a Western-blot of *Crysptosporidium* sporozoite/oocyst proteins. Fayer et al reported in 1990 that the antibodies in HBC were responsible for the protective activity of HBC *in vivo*. The experiments conducted by one of the inventors and others summarized here take Fayer's finding a step further by showing that antibodies to specific *Crysptosporidium* antigens are also responsible for the *in vitro* effect of the HBC Ig fraction. The inhibition in the *in vitro* assay correlated well with the effect of HBC and HBC Ig *in vivo*. Thus, the *in vitro* MDCK cell model stands validated as a model for detecting antibodies which are expected to be protective *in vivo*.

With the above validated model in hand, one of the inventors showed that antibodies raised to the fusion proteins of 3 of the antigens of the invention are significantly inhibitory in the *in vitro* MDCK cell model. This finding was then supported by similar results obtained with another epithelial cell line, MDBK cells. These observations indicate that the antibodies of the invention will be effective *in vivo*.

Thus, also part of this invention is a method of retarding, inhibiting or countering *Cryptosporidium* infection of a subject's cells comprising administering to a subject in need of such treatment an amount of the polypeptide of this invention capable of eliciting from the subject a host cell invasion and/or development inhibitory amount of anti-*Cryptosporidium* antibodies. Several *Cryptosporidium* polypeptides of this invention have been shown to elicit antibodies which inhibit *Cryptosporidium* infectivity and/or development inside the cell. In one preferred embodiment, the polypeptide of the invention, suitable for eliciting anti-*Cryptosporidium* antibodies in a mammal, comprises the S7, S24, S19, S34 or S2 polypeptides and other polypeptides encompassed by the corresponding entire proteins. More preferred are the S19, S34 and S2 polypeptides and other polypeptides encompassed by the corresponding entire proteins, and most preferred are S19 and S34 and other polypeptides encompassed by the corresponding entire proteins. Typically, a dose of about 0.02 to 10 mg is preferred, and more preferred is an amount of about 0.1 to 1 mg. However, other amounts are also suitable, as an artisan would know to adjust in accordance to weight and other variables such as the severity of the challenge.

This invention also provides a method of retarding, inhibiting or countering a *Cryptosporidium* infection of a subject's cells comprising administering to a subject in need of such treatment an amount of an antibody capable of binding to one or more of the polypeptides described above effective to retard the *Cryptosporidium* invasion of and/or development in the subject's cells. Antibodies selectively binding to several *Cryptosporidium* polypeptides have been shown by the inventors and others to inhibit *Cryptosporidium* infectivity *in vitro*. In one preferred embodiment, the antibody comprises anti-S7, anti-S24, anti-S19, anti-S34, or anti-S2 antibodies or mixtures thereof, other antibodies capable of selectively binding to other polypeptides encompassed by the corresponding entire

proteins. More preferred are the anti-S19, anti-S34, anti-S2 or anti-S24 antibodies and other antibodies capable of selectively binding to other polypeptides encompassed by the corresponding entire proteins and even more preferred are the anti-S19, anti-S34 and anti-S2 antibodies and other polypeptides encompassed by the corresponding entire proteins. Typically, the antibodies specific for *Cryptosporidium* may be administered in an amount of about 0.01 to 100 g, and more preferably about 1 to 35 g. However, other amounts may also be prescribed depending on the severity of the infection and other variables, as an artisan would know.

Both the polypeptide and the antibodies of this invention may be administered in a composition also comprising a carrier, preferably a biologically-acceptable carrier, and even more preferably a pharmaceutically-acceptable carrier. Examples of carriers are those described above. Formulations suitable for the administration of polypeptides and antibodies such as those described herein are known in the art, and need not be further described herein. Typically, other components stimulatory of immune response may be added as well as fillers, coloring, and the like.

Still part of this invention is a kit for the diagnosis of *Cryptosporidium* infection, that comprises

the polypeptide of this invention; and

instructions for use of the kit.

This kit may be utilized for the detection of endogenous antibodies produced by a subject that is afflicted with *cryptosporidiosis*. Even at the early stages where the parasite is commencing invasion of a subject's cells, some amount of *Cryptosporidium* specific antibody may be detected in serum.

Also provided herein is another *Cryptosporidium* diagnostic kit, that comprises

anti-*Cryptosporidium* antibodies having specificity for one of the polypeptides of this invention; and

instructions for use of the kit.

Thus, kit may be utilized for the detection of *Cryptosporidium* polypeptides, a sign that there is parasite present in the subject being tested.

In addition to the above, the kits may also comprise a control, anti-antibodies, protein A/G, and the like, suitable for conducting the different assays referred to above.

Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein for purpose of illustration only and are not intended to be limiting of the invention or any embodiment thereof, unless so specified.

EXAMPLES

Example 1: Parasites

Cryptosporidium sp. oocysts isolated from patients with AIDS at San Francisco General Hospital were used in the production of polyclonal and monoclonal antibodies and for molecular karyotype analysis.

Oocysts were isolated by resuspension of 1 vol. of feces with 2 vol. of a saturated NaCl solution. All subsequent procedures were done at 4 °C. After centrifugation at 1,000 g, the supernatant was recovered and the procedure repeated 3 times. The oocysts were recovered from the pooled supernatants by centrifugation, purified further in a 55%-27.5%-14% sucrose gradient at 1000 g for 20 min., and stored in PBS. Prior to use, the oocysts were sterilized by incubation in 15% commercial bleach, and washed by repeated centrifugation and re-suspension in PBS. The purified oocysts were excysted by incubation in Rpmi medium (gibco) with the addition of 0.75% sodium taurocholate (Sigma), pH 7, for 40-60 min. at 37°C.

Sporozoites were separated from unexcysted oocysts and debris by filtration through a polycarbonate 3 μm pore size membrane (Millipore).

Cryptosporidium oocysts from calves (Dr. Bruce Anderson, University of Idaho) were used for the isolation of DNA for the construction of the lambda gt11 genomic expression libraries. *Cryptosporidium parvum* oocysts of an AUCP-1 isolate (Dr. Byron Blagburn, Auburn University, Auburn, Alabama) propagated in Holstein calves were used for Western blots and indirect immunofluorescent antibody (IFA) studies.

Example 2: Preparation of Murine Polyclonal and Anti-oocyst / Sporozoite MAbs

10 week-old female BALB/c mice were immunized four times intraperitoneally with approximately $5x10^5$ sonicated *C.parvum* oocysts. The ascites were extracted and the antibodies isolated there- from. The polyclonal antibody fraction of the ascites was shown to react with the *C. parvum* sporozoite surface, the oocyst surface and internal antigens of the oocysts as assessed by an IFA as described in Petersen et al., Infect. Immun. 60(12):5132 (1992).

For monoclonal antibody production, mice were immunized intravenously with the supernatant from sonicated *C.parvum* oocysts three days before fusion as previously described by Kearney et al and Danforth et al (Kearney et al., J. Immunol. 123:1548(1979); Danforth et al., J. Parasitol. 68:1029(1982)). The hybridoma supernatants were used as the source of antibodies.

Six sporozoite monoclonal antibodies were obtained. The 1OC6, 7B3 and E6 monoclonal antibodies were determined to be of subclass IgG1 by an ELISA (Zymed) assay. The supernatants of the corresponding hybridoma cultures were used for IFA studies and Western blots.

Example 3: Immunoprecipitation Study

A *C.parvum* extract was prepared from a lysate using 2% SDS and 1% Triton X-100, and immunoprecipitated as described by Leech et al (Leech et al., J. Exp. Med. 159:1567(1984)). Monoclonal antibodies obtained in Example 2 above were added to the lysate and the resulting immune

complexes collected with protein A/G agarose beads. The *C. parvum* polypeptides from the immune complexes were separated on 5% SDS-PAGE gels and analyzed using a Western blot.

Example 4: Surface Iodination and Furhter Immunoprecipitation Studies

Sporozoites were washed twice by resuspension in Dulbecco-PBS with the addition of 1% glucose, taken to a final concentration of 10^9cells/ml, and radiolabeled with 300 μCi of ^{125}INa (Amersham, Arlington Heights, IL) in an Iodogen (Pierce, Rockford, IL) coated glass vial as previously described, with the exception that addition of 5 mM KI was omitted (Gardiner, P. R., et al., "Iodination and Identification of Surface Membrane Antigens in Procyclic *Trypanosoma rhodesiense*", J. Imm. 131(1):453 (1983)). Radiolabeled sporozoites were subsequently washed three times in RPMI medium with protease inhibitors as described above. After disruption of the labeled sporozoites by 5 cycles of freezing-thawing, the membrane pellet and the soluble fraction containing cytoplasmic proteins were collected. An aliquot of the membrane pellet was directly boiled in SB, and stored at -70 °C (sporozoite membrane proteins).

The remaining membrane material was divided into two radiolabeled samples. Prior to immunoprecipitation, one aliquot was extracted by boiling in SB as above, followed by addition of 9 vol. of NETT (0.15M NaCl; 5 mM EDTA; 0.5M Tris; 0.5% Triton X-100; pH 7.4) with 1% BSA (Sigma), 1% Triton-X 100, and protease inhibitors (SDS solubilized membranes). The other radiolabeled membrane sample was extracted directly with 3 vol. of NETT with 1% BSA, 1% Triton X-100, and protease inhibitors (Triton X-100 solubilized membranes). Both samples were precleared by addition of 1μl SHAM-HBC Ig followed by overnight incubation at 4°C. Subsequently, 200 μl of Protein G-Sepharose 4B beads were added and the samples rocked for 1 hr. at room temperature. After centrifugation at 10,000

g, HBC Ig affinity bound to Protein G- Sepharose 4B beads (300 µl) were added to the supernatants and the samples rocked for an additional 2 hrs. at 37 °C.

The immunoprecipitates were washed sequentially with NETT buffer alone and NETT containing 1% BSA (Sigma) or 500 mM NaCl, then boiled in SB and stored at -70 °C. Proteins were separated in 5-15% gradient gels by SDS-PAGE, and processed for autoradiography using X-Omat film (Kodak). Iodination controls consisted of tri-chloroacetic acid (TCA) precipitates of the soluble fraction containing sporozoite cytoplasmic proteins which were also processed as described above.

Example 5: Preparation of HBC

Hyperimmune bovine colostrum (HBC lot# 40529) was obtained from ImmuCell Corporation (Portland, Maine) and was prepared by repeated parenteral immunization of Holstein cows during pre-parturition with partially excysted *C. parvum* oocysts. Immunogens were emulsified in Freund's adjuvant.

SHAM-HBC (lot # 41038) was prepared after immunization with a commercial herd health vaccine which was also given to cows immunized with *C. parvum* to prepare HBC (lot# 40529).

Colostra were collected using standard dairy practices and frozen. A 0.45 µm filtered, lyophilized colostral whey immunoglobulin (Ig) preparation, free of low molecular weight solutes, was prepared from pooled colostra from several immunized animals. Colostra were partially purified to obtain antibody products highly enriched for IgG (HBC Ig and SHAM-HBC Ig).

Colostral Ig concentrates were prepared using large scale production methods developed at ImmuCell Corp. Briefly, a pasteurized whey preparation of colostrum was prepared to eliminate the majority of caseins and fat. This preparation was then subjected to a series of ultrafiltration and microfiltration steps to remove small molecular weight solutes such as lactose and some whey

proteins/peptides as well as particulate matter including residual fat and caseins. The resulting concentrate was filtered, dried and shown to be stable at room temperature. These preparations were greater than 85% protein by weight, and greater than 55% IgG on a protein basis. The degree of IgG purification was at least 2 fold.

The HBC of lot #40529 was used in the animal protection studies of Example 32 below, and the HBC Ig (50 mg/ml IgG) of the same lot was used in the *in vitro* inhibition of development studies of Example 34 below. Anti-*Cryptosporidium* Ab titers were determined independently several times for each colostrum preparation. HBC Ig (lot # 40529) had an average anti-*Cryptosporidium* antibody titer of 1/176,000 U/ml for a 43 $\mu g/\mu l$ IgG concentration by ELISA. SHAM-HBC Ig (lot # 41038) had an approximately ten fold lower average antibody titer to *Cryptosporidium* antigens by ELISA (17,000 U/ml for a 45 $\mu g/\mu l$ IgG concentration), probably due to natural infection of the animals in the field.

Example 6: Assessment of *in vivo* Efficacy of HBC

Four newborn, colostrum deprived Holstein calves were fed 100 ml HBC (lot# 40529) plus 2 quarts commercial milk replacer at 4 hrs. of age (treated group). Similarly, four calves were fed non-immune colostrum (control group) and all other parameters were equal. All 8 animals were challenged at 12 hrs. of age with $5x10^6$ oocysts of *C. parvum*. All animals were fed 100 ml of SHAM-HBC or HBC every 24 hrs. and 2 qt milk replacer every 12 hrs.

Clinical observations of diarrhea and dehydration were made every 12 hrs. over a 7 day interval on all calves, and fecal samples were taken every 12 hrs. Fecal and dehydration scores were tabulated from days 5-7, the days of peak patency. Oocyst shedding was tabulated over days 5-9 post infection in 3/4 treated animals and 2/4 controls. Samples from the remaining animals were not available. Oocyst shedding was measured by mixing 1 vol. fecal sample with 4 vol. Sheather's solution and enumerating the

refractive oocysts in a hemocytometer. Confirmation of the oocyst counts was performed with a commercial immuno-fluorescence kit utilizing a monoclonal anti-oocyst antibody (Merifluor, Meridian Diagnostics, Cincinnati, OH).

The efficacy of the immune colostrum preparation for protecting the treated calves from *C.parvum* infection was demonstrated in statistically significant differences between treated and control animals in cumulative fecal scores ($p < 0.01$ by one tailed t test) and dehydration scores ($p<0.01$ by one tailed t test).

No dehydration occurred in the treated group whereas all of the calves in the control group showed some signs of dehydration. The oocyst output was dramatically reduced in the treated group ($<10^3$ oocysts per total fecal output, the limit of detection) when compared to the control group (geometric mean oocyst output = 5.62×10^8).

These results clearly show that the immune colostrum treatment was effective to reduce the initial colonization by *C.parvum* parasites as well as to suppress the intestinal proliferation of the *C.parvum* parasites which were not initially neutralized.

Example 7: Preparation of *C.parvum* DNA Expression Libraries

Two *C. parvum* lambda gt11 genomic expression libraries were constructed. A restriction fragment expression library described by Kim et al and Nelson et al was utilized (Kim et al., Mol. Biochem. Parasitol., 50:105(1992); Nelson et al., J. Protozool. 386:52 (1991)).

A second expression library was constructed using aliquots of *C. parvum* DNA which was partially digested with DNase I in 33 mM Tris-HCI pH 7.4, 5 mM $CaCl_2$ for 15 min. Briefly, EDTA was added to a concentration of 20 mM, and aliquots removed and extracted once with phenol, and pooled. The pooled sample was extracted twice with phenol, twice with chloroform:isoamyl alcohol and once with diethyl ether and then ethanol precipitated. The DNA was subsequently treated with Klenow and T4 DNA polymerases to repair staggered ends, with EcoR1 methylase to protect

internal EcoRI sites and ligated to EcoRl linkers (pCCGAATTCGG) as is known in the art (Petersen et al., Infect. Immunol. (1992), supra). The polymeric linkers were removed by EcoRI digestion, and the DNA was purified by exclusion chromatography on Sephadex G-I00. Any DNA eluting in the void volume was ethanol precipitated and ligated to EcoRl-cleaved, alkaline phosphatase-treated lambda gt11 arms, packaged *in vitro*, plated and amplified in *E. coli* strain Y1090. The resulting library was 70% recombinant and contained 1.2 million independent clones.

Example 8: Screening of the Two Expression Libraries

The two libraries were screened with polyclonal anti-*C. parvum* oocyst/sporozoite antibodies obtained as described in Example 2 and positive clones were plaque purified as described by Petersen et al., Infect. Immun. (1992), supra. 275,000 plaques from the amplified restriction fragment library were screened. 52 of those clones expressing fusion proteins were identified and purified. In addition, 225,000 plaques of the DNase library were screened and 5 of those clones were identified as positive and purified.

Example 9: Separation of Antibody Subgroups Specifically Binding Polypeptides from 5 Distinct Groups

Antibodies specifically binding to the 5 distinct sporozoite polypeptide groups from IPTG-induced confluent plaque lifts of purified lambda gt11 clones were selected by affinity chromatography from a polyclonal anti-*C. parvum* oocyst/sporozoite antibody preparation on the respective plaquelifts. The antibodies were then eluted with 10 mM glycine pH 2.6, and 150 mM NaCl as described by Beall and Mitchell (Beall and Mitchell, J. Immunol. Meth. 86:217(1986); Coppel et al., Protocols in Molec. Parasitol., in Methods in Molecular Biology Series, Humana Press, NJ (1993); Petersen et al., Mol. Biochem. Parasitol. 42:189 (1990)). Antibodies were isolated from each of the 57 expressing clones.

Example 10: Sibling Analysis of Recombinant Clones

Approximately 400 plaque forming units (pfu) of each of the lambda gt11 purified clones and wild type lambda gt11 were individually dotted in a grid pattern onto a lawn of Y1090 on 152 mm Petri dishes. The dishes were incubated for 3 hrs at 37°C, and then a nitrocelluose filter saturated with 50 mm IPTG was applied to each lawn and allowed to incubate overnight. The filters were then removed and washed three times in TBSTA (10 mM Tris-HCI, pH 8.0; 150 mM NaCl; 0.05% Tween; 0.2% Na azide), and blocked with 1% BSA-TBSTA. Each of the 57 filters was incubated with a single antibody sample prepared from one of the clones for 2 hrs., washed 3 times with TBSTA and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Promega) for 1hr. The filters were then developed with the colorometric reagents nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

Each of the 57 recombinants was thus segregated into 1 of 5 sibling groups based on the immunologic cross-reactivity of their encoded antigens. For example, antibodies isolated from clone S34 bound to fusion proteins expressed by clones S34, S38, S41 and S57 but not to the proteins expressed by the other 53 recombinants or wild type lambda gt11 clones. Correspondingly, antibodies isolated from clone S38 bound to clones S34, S38, S41 and S57 but to none of the other clones. The number of clones in each of the sibling groups is indicated in column 2 of Table 3 below.

Table 3: Sporozoite Proteins Encoded By Cloned *C. parvum* Gene

Sibling Group	Number of Clones	IFA Localization	MW of Protein
S34	4	1/2 sporozoite	>900
S19	2	apical	68/95
S2	4	flocculant	45
S7	1	diffuse	23
S24	5	diffuse	15/32-35

Example 11: Indirect IFA Localization of Endogenous Antigens in Sporozoites and Oocysts

Slides containing air-dried, acetone-fixed sporozoites and oocysts were incubated with antibodies isolated as described in Example 8 above for 1hr in a humidified chamber, washed with phosphate buffered saline, pH 7.4, and incubated with affinity purified goat anti-mouse IgG/IgA/IgM conjugated with fluorescein isothiocyanate (Zymed). The slides were counterstained with Evan's blue, and coverslips were applied onto them. The slides were then observed and photographed with a microscope (Nikon Optiphot) equipped for immunofluorescence with fluorescein.

A diffuse IFA staining pattern of sporozoites was produced with the antibodies. This pattern indicated which of the sibling groups contained clones encoding *C. parvum* polypeptides that were candidates to reside in the sporozoite pellicle. The IFA staining of the anterior portion of the sporozoites, where rhoptries, micronemes and dense granules are located, served to identify candidate apical organelle polypeptides.

The antibodies eluted from clones of the S34 group reacted with the anterior one-half of fixed sporozoites on an IFA whereas antibodies eluted from clones of the S19 group reacted with the anterior tip of the sporozoite in a very localized manner and antibodies eluted from clones of the S2 group exhibited a flocculant pattern over the

sporozoites. The antibodies eluted from the single S7 clone, and members of the S24 group on the other hand, reacted diffusely with the fixed sporozoite on IFA but not with the oocyst.

The localization of antigens specific for the antibodies eluted from cloned antigens is indicated in column 3 of Table 3 above.

Example 12: Identification of Endogenous Antigens Encoded by Cloned Gene Fragments

One hundred million oocysts were suspended in 500 µl of a protease inhibitor cocktail containing 100 µM leupeptin (Sigma), 100 µM chymostatin (Sigma), 100 µM pepstatin (Sigma), 100 µM trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (Sigma), 100 µM phenylmethylsulfonyl fluoride (Sigma), 50 mM N alpha-para-tosyl-L lysine chloromethyl ketone (Sigma), 150 mM NaCl and 500 mM EDTA, pH 8.0, and lysed by 5 freeze-thaw cycles. Western blots were prepared as described by Petersen et al (Petersen et al., Mol. Biochem. Parasitol. 42:189(1990)), and incubated with the individual eluted antibodies.

Alkaline phosphatase labeled second antibodies and the substrates described above, were used to detect antigens which bound to the eluted antibody. The Western blots were scanned with a Hewlett Packard flatbed scanning densitometer in reflectance mode prior to photography. Nebulin (900 kD; Stedman et al., Genomics 2:1(1988)), and titin (2500 kD, Kurzban and Wang, Biochem. Biophys. Res. Commun. 150:1155(1989)), were used as high molecular weight standards. These standards were provided by Dr. Kuan Wang, University of Texas, Austin.

The antibodies eluted from the five sibling groups encoding sporozoite proteins recognized distinct polypeptides on Western blots. The antibodies eluted from clones of the S34 group bound to a polypeptide of apparent molecular weight >900 kD on Western blots of oocyst/sporozoite proteins. This polypeptide migrated

faster than titin at 2500 kD and slower than nebulin at 900 kD.

Antibodies eluted from clones of the S19 group bound to a polypeptide apparent molecular weight 68 kD, and weakly bound to a polypeptide of molecular weight 95 kD. The antibodies eluted from the S2 group bound to a 45 kD polypeptide. The antibodies eluted from the S7 group bound to a 23 kD polypeptide, and antibodies eluted from the S24 group bound to a 15 kD polypeptide doublet and to a 32-35 kD polypeptide doublet on Western blots.

Western blots probed with the murine oocyst/sporozoite antibodies from which the eluted antibodies were isolated indicated that the antibodies reacted with many different oocyst/sporozoite antigens. Control eluted antibodies prepared from wild type lambda gt11 clones which only express beta-galactosidase showed substantially no binding to oocyst/sporozoite polypeptides. This clearly indicates that the elution process yielded highly specific antibody. It is unclear if the fact that multiple polypeptide bands were identified by the S19 and S24 eluted antibodies is due to antigenic cross-reactivity or post-translational processing. Although parasite proteolysis occurring prior to lysis in protease inhibitors cannot be ruled out it appears unlikely since use of the protease cocktail routinely allowed the detection of the polypeptide of apparent molecular weight >900 kD of the S34 family in undegraded form. This indicates that protein degradation is minimal.

The molecular weights of endogenous polypeptides for which the antibodies eluted from the cloned antigens have specificity is indicated in column 4 of Table 3 above.

Example 13: Detection of High Molecular Weight *C. parvum* Polypeptide with HBC IgG

A whole oocyst/sporozoite lysate was electrophoresed in a 5% SDS-PAGE gel and Western blotted with HBC IgG. At least 10 polypeptides were identified. Because of the compression effect of the polypeptides in the region below

the 46 kD band, the number of polypeptides in that region bound by HBC is uncertain. Sham IgG did not identify any proteins. An antigen identified by HBC IgG which generated a very strong signal co-migrated with a very high molecular weight antigen recognized by MAb 10C6.

Immunoprecipitation of Triton X-100 soluble oocyst/sporozoite proteins with monoclonal antibody 10C6 followed by SDS-PAGE and Western blot with HBC Ig confirmed that the antigenic sites reside on the same molecule. Control lanes indicate that additional antigens are heavy and light chains from antibodies in the fractionated immune complexes. Coomassie blue staining of SDS-PAGE gels suggested that the >900 kD antigen predominates over other proteins in oocyst/sporozoite lysates.

Studies with surface labeled parasites immunoprecipitated with HBC Ig indicated that the >900 kD antigen is surface exposed.

Example 14: Binding of Sporozoite Specific Monoclonal Antibodiesto > 900 kD Apparent MW Polypeptide

Immunoprecipitation of Triton X-100 extracted oocyst/sporozoite polypeptides with monoclonal antibody 10C6 followed by Western blot with monoclonal antibodies 10C6, 7B3 and E6 evidenced that all three monoclonal antibodies bound to the same molecular weight >900 kD band. Thus, three of six monoclonal antibodies developed to sporozoites had specificities directed at epitopes of this antigen. In addition, three monoclonal antibodies out of eight raised against intracellular organisms recognize this same antigen suggesting that it, or a crossreacting antigen, is also present in the merozoites.

The 7B3 monoclonal antibody also identified a 38 kD apparent molecular weight antigen on a Western blot of whole oocyst/sporozoite lysates but not on Western blots of whole sporozoite lysates. This suggests that a molecule from oocysts that is insoluble in Triton-X-100 also reacts with monoclonal antibody 7B3, the monoclonal antibody

reacting with the anterior portion of fixed sporozoites on an IFA. When the 10C6 monoclonal antibody was used in an IFA of fixed intracellular merozoites cultivated in MDCK cells, a linear pattern was obtained that suggested localization at the pellicle of the elongated merozoite. This indicates that the epitope for which the monoclonal antibody 10C6 has specificity is also present in this second invasive stage.

Example 15: Inhibition of *C. parvum* Infection by GP900 Polypeptide Specific Monoclonal Antibody

Sporozoites were obtained from oocysts isolated from calves infected with the AUCP-1 strain of *C. parvum*. The sporozoites were cultured *in vitro* on Madin-Darby canine kidney (MDCK) cells as described by Gut et al (Gut et al., J. Protozool. 386:56 (1991)).

Briefly, freshly excysted, untreated, sporozoites were allowed to invade monolayers of MDCK cells in a quantitative fashion. The sporozoites underwent cycles of asexual and sexual development. In another experiment, when viable sporozoites were pre-incubated for 30 minutes with monoclonal antibody 10C6, it inhibited the invasion of MDCK cells by greater than 90% when compared to control antibody.

GP900 thus appears to be an effective target for passive or active antibody immunotherapy. In addition, monoclonal antibody 10C6 was shown to be an effective antibody against GP900.

Example 16: The >900 kD App. MW C. parvum Polypeptide is N-glycosylated

A Western blot analysis of oocyst/sporozoite polypeptides immunoprecipitated with monoclonal antibody 10C6 indicated that the S34 eluted antibodies bind to the >900kD polypeptide that is also immunoprecipitated by monoclonal antibody 10C6. Thus, the S34 polypeptide encodes a portion of the >900kD app. MW polypeptide. The >900kD polypeptide is a highly immunogenic molecule that binds to the 10C6, 7B3 and E6 monoclonal antibodies and to HBC IgG.

A *C.parvum* lysate was incubated overnight at 37°C with N-glycosidase F (Boehringer Mannheim, EC 3.2.2.18) according to Boehringer Mannheim instructions, and then electrophoresed in a 5 % SDS PAGE gel and Western blotted. To control proteolysis during the incubation, the *C. parvum* lysate was incubated under the same conditions in the absence of N-glycosidase F enzyme.

The oocyst/sporozoite proteins were treated with N glycosidase F, and then electrophoresed on a 5 % SDS PAGE gel and Western blotted with the 10C6, 7B3 and E6 monoclonal antibodies. None of the monoclonal antibodies detected an antigen on the Western blots. However, the S34-eluted antibodies recognized multiple *C. parvum* N-deglycosylated proteins of apparent molecular weight <200 kD.

Taken together these results indicate the following.

1) The monoclonal antibodies react with an epitope that requires intact N-glycosylation.
2) The polypeptide is thus a glycopeptide.
3) The apparent molecular weight of the N-deglycosylated polypeptide is <190 kD.
4) Immunoprecipitation of Triton X-100 soluble oocyst/sporozoite polypeptides and SDS extraction of the Triton X-100 insoluble pellet indicate the >900kD apparent molecular weight glycoprotein to be mostly Triton X-100 soluble.

Example 17: A Single Copy Gene Encodes >900 kD Apparent Molecular Weight Glycoprotein

The genomic Southern analysis and ^{32}P dATP labeling of a 1.2 kb S34 DNA insert cloned into pGem was carried out as described by Petersen et al. (Petersen et al., Mol. Biochem. Parasitol. 36:61(1989)).

After hybridization, the membranes were washed with 0.1 x SSPE, 0.25% Sarkosyl at 65°C and an autoradiogram obtained. The autoradiogram of the genomic Southern blot was scanned with a Hewlett Packard flatbed scanning densitometer prior to photography. The chromosomes were transferred to membranes and molecular karyotype analysis was performed probing with pGemS34 as described by Kim et

al. (Kim, et al., Mol. Biochem. Parasitol. 50:105 (1992)).

Genomic Southern analysis showed the 1.2 kb S34 insert to hybridize with single BstE II, Apa I, Bcl I, Cla I and SSp I fragments of approximate size of 9.0, >23, >23, 6, and 4 kb, respectively, and with two restriction fragments generated by Ba II.

These results show the >900 kD glycoprotein to be encoded by a single copy gene. Molecular kararoyotypic analysis also showed that the gene is located on the largest detected *C. parvum* chromosome, a chromosome of approximately 1,400 kb in both bovine and human isolates.

Example 18: DNA Sequence of Clone S34

The DNA nucleotide sequence of the *C. parvum* insert of clone S34 was determined by chain termination DNA sequencing (Sanger, F., et. al., Proc. Nat. Acad. Sci. (USA) 74:5463 (1977)). *Cryptosporidium* DNA inserts were excised from lambda gt11 with EcoRI and ligated into m13 (Messing, J., Methods in Enzymology 101:20 (1983)) prior to sequencing. Sequencing was carried out exactly as detailed on pages 6-9 of the Sequenase^{R} Version 2.0 protocol provided with the commercially available enzyme, Sequenase^{R} (United States Biochemical, pages 6-9). Labeled m13 mixtures were separated by denaturing gel electrophoresis using a Bio-Rad sequencing cell.

The DNA sequence obtained and that of its complementary strand are provided in Table 4 below as well as the 6 possible amino acid sequences deduced therefrom.

Table 4

DNA and amino acid sequences of clone S34

1/1 31/11

glu phe pro asp arg ser leu asp phe thr ile pro pro val ala gly his asn ser cys
arg val pro arg AMB lys phe gly phe his asn ser ser ser ser trp pro OCH gln leu
ala ser ser gln ile glu val trp ile ser gln phe leu gln AMB leu ala ile thr ala
GCG AGT TCC CAG ATA GAA GTT TGG ATT TCA CAA TTC CTC CAG TAG CTG GCC ATA ACA GCT
CGC TCA AGG GTC TAT CTT CAA ACC TAA AGT GTT AAG GAG GTC ATC GAC CGG TAT TGT CGA
arg thr gly leu tyr phe asn pro asn OPA leu glu glu leu leu gln gly tyr cys ser
ser asn gly ser leu leu lys ser lys val ile gly gly thr ala pro trp leu leu gln
leu glu trp ile ser thr gln ile glu cys asn arg trp tyr ser ala met val ala thr

61/21 91/31

ser ile ile val gly val ser gly asp gly lys ile his val ser pro tyr gly ser lys
phe asn asn ser trp cys glu arg arg trp lys asn ser arg lys pro ile arg phe OCH
val gln OCH AMB leu val OPA ala ala met glu lys phe thr OCH ala his thr val leu
GTT CAA TAA TAG TTG GTG TGA GCG GCG ATG GAA AAA TTC ACG TAA GCC CAT ACG GTT CTA
CAA GTT ATT ATC AAC CAC ACT CGC CGC TAC CTT TTT AAG TGC ATT CGG GTA TGC CAA GAT
asn leu leu leu gln his ser arg arg his phe phe glu arg leu gly met arg asn AMB
glu ile ile thr pro thr leu pro ser pro phe ile OPA thr leu gly tyr pro glu leu
OPA tyr tyr asn thr his ala ala ile ser phe asn val tyr ala trp val thr arg leu

121/41 151/51

asp val ser leu ile ser ala pro ile gln pro ser glu leu phe asn glu val tyr cys
gly cys leu ser asn lys cys ser asn thr thr phe OPA val ile gln OPA ser leu leu
arg met ser leu OCH OCH val leu gln tyr asn leu leu ser tyr ser met lys phe ile
AGG ATG TCT CTC TAA TAA GTG CTC CAA TAC AAC CTT CTG AGT TAT TCA ATG AAG TTT ATT
TCC TAC AGA GAG ATT ATT CAC GAG GTT ATG TTG GAA GAC TCA ATA AGT TAC TTC AAA TAA
pro his arg glu leu leu his glu leu val val lys gln thr ile OPA his leu lys asn
ser thr glu arg ile leu ala gly ile cys gly glu ser asn asn leu ser thr OCH gln
ile asp arg AMB tyr thr ser trp tyr leu arg arg leu OCH glu ile phe asn ile ala

181/61 211/71

asp thr cys thr ala lys tyr gly ala ile his ser gly tyr gln thr ser ala asp phe
arg his leu tyr cys glu val trp cys asn ser leu trp ile ser asn phe ser OPA phe
ala thr leu val leu arg ser met val gln phe thr leu asp ile lys leu gln leu ile
GCG ACA CTT GTA CTG CGA AGT ATG GTG CAA TTC ACT CTG GAT ATC AAA CTT CAG CTG ATT
CGC TGT GAA CAT GAC GCT TCA TAC CAC GTT AAG TGA GAC CTA TAG TTT GAA GTC GAC TAA
arg cys lys tyr gln ser thr his his leu glu ser gln ile asp phe lys leu gln asn
ser val gln val ala phe tyr pro ala ile OPA glu pro tyr OPA val glu ala ser lys
val ser thr ser arg leu ile thr cys asn val arg ser ile leu ser OPA ser ile glu

Table 4 (cont.)

241/81 271/91
val thr thr leu pro thr thr thr gly ala ala gly gln pro thr thr thr thr thr gly
arg asn asn thr ser tyr tyr asn trp ser arg arg thr thr asn asn tyr tyr asn trp
ser OCH gln his phe leu leu gln leu glu pro gln asp asn gln gln leu leu gln leu
TCG TAA CAA CAC TTC CTA CTA CAA CTG GAG CCG CAG GAC AAC CAA CAA CTA CTA CAA CTG
AGC ATT GTT GTG AAG GAT GAT GTT GAC CTC GGC GTC CTG TTG GTT GTT GAT GAT GTT GAC
arg leu leu val glu AMB AMB leu gln leu arg leu val val leu leu AMB AMB leu gln
thr val val ser gly val val val pro ala ala pro cys gly val val val val val pro
tyr cys cys lys arg ser cys ser ser gly cys ser leu trp cys ser ser cys ser ser

301/101 331/111
ser pro ser lys pro thr thr thr thr thr ile OCH gly asn asn asn his asn asn ser
lys ser lys gln thr asn tyr tyr tyr his tyr leu arg gln gln gln pro gln gln leu
glu val gln ala asn gln leu leu leu pro leu ser lys ala thr thr thr thr thr thr
GAA GTC CAA GCA AAC CAA CTA CTA CTA CCA CTA TCT AAG GCA ACA ACA ACC ACA ACA ACT
CTT CAG GTT CGT TTG GTT GAT GAT GAT GGT GAT AGA TTC CGT TGT TGT TGG TGT TGT TGA
phe asp leu cys val leu AMB AMB AMB trp AMB arg leu cys cys cys gly cys cys ser
leu gly leu leu gly val val val val val ile AMB pro leu leu leu trp leu leu glu
thr trp ala phe trp ser ser ser gly ser asp leu ala val val val val val val arg

361/121 391/131
OCH ser asn his tyr asn asn asn ser lys thr asn asn asn asn asn asn lys gly ser
leu ile gln ser leu gln gln gln leu lys asn gln gln gln gln gln gln gln arg phe
leu asn pro ile ile thr thr thr thr gln lys pro thr thr thr thr thr thr lys val
CTT AAT CCA ATC ATT ACA ACA ACA ACT CAA AAA CCA ACA ACA ACA ACA ACA ACA AAG GTT
GAA TTA GGT TAG TAA TGT TGT TGT TGA GTT TTT GGT TGT TGT TGT TGT TGT TGT TTC CAA
lys ile trp asp asn cys cys cys ser leu phe trp cys cys cys cys cys cys leu asn
OCH asp leu OPA OCH leu leu leu glu phe val leu leu leu leu leu leu pro glu
leu gly ile met val val val val OPA phe gly val val val val val val phe thr gly

421/141 451/151
arg OCH ala thr asn ser his asn asn asn asn ile lys ala asn ser tyr asn asn asn
gln val ser his gln AMB pro gln gln gln gln his OCH ser gln AMB leu gln gln gln
pro gly lys pro pro ile ala thr thr thr thr thr leu lys pro ile val thr thr thr
CCA GGT AAG CCA CCA ATA GCC ACA ACA ACA ACA ACA TTA AAG CCA ATA GTT ACA ACA ACA
GGT CCA TTC GGT GGT TAT CGG TGT TGT TGT TGT TGT AAT TTC GGT TAT CAA TGT TGT TGT
trp thr leu trp trp tyr gly cys cys cys cys cys OCH leu trp tyr asn cys cys cys
leu tyr ala val leu leu trp leu leu leu leu met leu ala leu leu OCH leu leu leu
pro leu gly gly ile ala val val val val val asn phe gly ile thr val val val val

Table 4 (cont.)

481/161 511/171
asn lys ser asn asn asn asn asn asn asn ser ala asn asp asn tyr tyr tyr gln glu
gln gln lys gln gln gln gln gln gln gln gln cys gln arg gln leu leu leu pro arg
thr thr lys ala thr thr thr thr thr thr thr val pro thr thr thr thr thr lys
ACA ACA AAA GCA ACA ACA ACA ACA ACA ACA ACA GTG CCA ACG ACA ACT ACT ACT ACC AAG
TGT TGT TTT CGT TGT TGT TGT TGT TGT TGT TGT CAC GGT TGC TGT TGA TGA TGA TGG TTC
cys cys phe cys cys cys cys cys cys cys cys his trp arg cys ser ser ser gly leu
leu leu leu leu leu leu leu leu leu leu leu ala leu ser leu AMB AMB AMB trp ser
val phe ala val val val val val val val thr gly val val val val val leu leu

541/181 571/191
arg arg asn asp asn asn asn asp thr ile thr OPA tyr arg OPA his OPA asn tyr thr
glu thr lys OPA gln gln gln arg his his tyr leu ile ser val thr leu lys leu his
arg asp glu met thr thr thr thr thr pro leu pro asp ile gly asp ile glu ile thr
AGA GAC GAA ATG ACA ACA ACA ACG ACA CCA TTA CCT GAT ATC GGT GAC ATT GAA ATT ACA
TCT CTG CTT TAC TGT TGT TGT TGC TGT GGT AAT GGA CTA TAG CCA CTG TAA CTT TAA TGT
ser val phe his cys cys cys arg cys trp OCH arg ile asp thr val asn phe asn cys
leu arg phe ser leu leu leu ser val met val gln tyr arg his cys gln phe OCH val
ser ser ile val val val val val gly asn gly ser ile pro ser met ser ile val gly

601/201 631/211
asn pro asn OPA lys asp val gly OCH val his lys asn asp leu OPA leu OCH gln trp
gln ser gln leu lys arg cys trp ile ser thr gln glu OPA phe met thr ile thr val
pro ile pro ile glu lys met leu asp lys tyr thr arg met ile tyr asp tyr asn ser
CCA ATC CCA ATT GAA AAG ATG TTG GAT AAG TAC ACA AGA ATG ATT TAT GAC TAT AAC AGT
GGT TAG GGT TAA CTT TTC TAC AAC CTA TTC ATG TGT TCT TAC TAA ATA CTG ATA TTG TCA
trp asp trp asn phe leu his gln ile leu val cys ser his asn ile val ile val thr
leu gly leu gln phe ser thr pro tyr thr cys leu phe ser lys his ser tyr cys his
ile gly ile ser phe ile asn ser leu tyr val leu ile ile OCH ser AMB leu leu pro

661/221 691/231
phe ile ile arg leu OCH OPA OPA thr asn ser arg phe ser ser arg thr asn ser OPA
val tyr tyr AMB thr leu met met asn gln phe gln val leu lys gln asp lys AMB leu
gly leu leu leu asp ser asn asp glu pro ile pro gly ser gln ala gly gln ile ala
GGT TTA TTA TTA GAC TCT AAT GAT GAA CCA ATT CCA GGT TCT CAA GCA GGA CAA ATA GCT
CCA AAT AAT AAT CTG AGA TTA CTA CTT GGT TAA GGT CCA AGA GTT CGT CCT GTT TAT CGA
thr OCH OCH OCH val arg ile ile phe trp asn trp thr arg leu cys ser leu tyr ser
asn ile ile leu ser AMB his his val leu glu leu asn glu leu leu val phe leu gln
lys asn asn ser glu leu ser ser gly ile gly pro glu OPA ala pro cys ile ala ser

Table 4 (cont.)

721/241 751/251
tyr lys gln phe ile pro arg phe lys leu thr arg val leu val tyr gln leu ile gln
ile gln ala ile tyr ser gln val gln thr his lys ser thr gly leu pro ile asp pro
asp thr ser asn leu phe pro gly ser asn ser gln glu tyr trp phe thr asn OPA ser
GAT ACA AGC AAT TTA TTC CCA GGT TCA AAC TCA CAA GAG TAC TGG TTT ACC AAT TGA TCC
CTA TGT TCG TTA AAT AAG GGT CCA AGT TTG AGT GTT CTC ATG ACC AAA TGG TTA ACT AGG
ile cys ala ile OCH glu trp thr OPA val OPA leu leu val pro lys gly ile ser gly
tyr leu cys asn ile gly leu asn leu ser val leu thr ser thr OCH trp asn ile trp
val leu leu lys asn gly pro glu phe glu cys ser tyr gln asn val leu gln asp leu

781/261 811/271
trp leu val phe his leu ile gln asn gln val ile AMB tyr ile his ile pro ile lys
met val gly leu pro phe asp pro lys ser gly asn leu val his pro tyr thr asn gln
asn gly trp ser ser ile OPA ser lys ile arg OCH phe ser thr ser ile tyr gln ser
AAT GGT TGG TCT TCC ATT TGA TCC AAA ATC AGG TAA TTT AGT ACA TCC ATA TAC CAA TCA
TTA CCA ACC AGA AGG TAA ACT AGG TTT TAG TCC ATT AAA TCA TGT AGG TAT ATG GTT AGT
ile thr pro arg gly asn ser gly phe asp pro leu lys thr cys gly tyr val leu OPA
his asn thr lys trp lys ile trp phe OPA thr ile OCH tyr met trp ile gly ile leu
pro gln asp glu met gln asp leu ile leu tyr asn leu val asp met tyr trp asp phe

841/281 871/291
gln cys leu val tyr arg tyr his ile leu leu leu arg ile OPA gln leu ile leu met
thr met ser gly leu ser val ser tyr leu ala ala lys asn leu thr val asp thr asp
asn asn val trp phe ile gly ile ile ser cys cys OCH glu phe asp ser OPA tyr OPA
AAC AAT GTC TGG TTT ATC GGT ATC ATA TCT TGC TGC TAA GAA TTT GAC AGT TGA TAC TGA
TTG TTA CAG ACC AAA TAG CCA TAG TAT AGA ACG ACG ATT CTT AAA CTG TCA ACT ATG ACT
val ile asp pro lys asp thr asp tyr arg ala ala leu phe lys val thr ser val ser
cys his arg thr OCH arg tyr OPA ile lys ser ser leu ile gln cys asn ile ser ile
leu thr gln asn ile pro ile met asp gln gln AMB ser asn ser leu gln tyr gln his

901/301 931/311
lys leu arg phe thr asn OPA tyr thr his trp leu pro ile gly ser ser gln phe asp
glu thr thr val tyr gln leu ile his ser leu val thr his trp ile gln ser val OPA
OPA asn tyr gly leu pro ile asp thr leu thr gly tyr pro leu asp pro val ser leu
TGA AAC TAC GGT TTA CCA ATT GAT ACA CTC ACT GGT TAC CCA TTG GAT CCA GTC AGT TTG
ACT TTG ATG CCA AAT GGT TAA CTA TGT GAG TGA CCA ATG GGT AAC CTA GGT CAG TCA AAC
ser val val thr OCH trp asn ile cys glu ser thr val trp gln ile trp asp thr gln
phe ser arg asn val leu gln tyr val OPA gln asn gly met pro asp leu OPA asn ser
phe AMB pro lys gly ile ser val ser val pro OCH gly asn ser gly thr leu lys ile

Table 4 (cont.)

961/321 991/331

ser val gln ser arg asn trp OPA ile val cys pro ile ser asp glu ile met asn gly
phe arg ser ile gln lys leu val asn cys leu ser asn ile arg OPA asp asn glu trp
ile pro phe asn pro glu thr gly glu leu phe val gln tyr gln met arg OCH OPA met
ATT CCG TTC AAT CCA GAA ACT GGT GAA TTG TTT GTC CAA TAT CAG ATG AGA TAA TGA ATG
TAA GGC AAG TTA GGT CTT TGA CCA CTT AAC AAA CAG GTT ATA GTC TAC TCT ATT ACT TAC
asn arg glu ile trp phe ser thr phe gln lys asp leu ile leu his ser leu ser his
glu thr OPA asp leu phe gln his ile thr gln gly ile asp ser ser ile ile phe pro
gly asn leu gly ser val pro ser asn asn thr trp tyr OPA ile leu tyr his ile ser

1021/341 1051/351

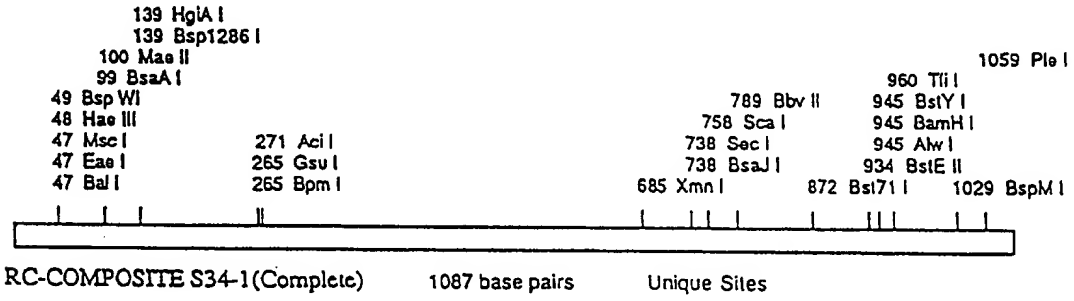
thr ile ala gly ile val ser gly ile ser ala ser glu ser leu leu ser gln lys ser
asn asn cys arg tyr cys phe arg asn phe cys lys OPA val ile ile ile ser glu ile
glu gln leu gln val leu phe gln glu phe leu gln val ser his tyr tyr leu arg asn
GAA CAA TTG CAG GTA TTG TTT CAG GAA TTT CTG CAA GTG AGT CAT TAT TAT CTC AGA AAT
CTT GTT AAC GTC CAT AAC AAA GTC CTT AAA GAC GTT CAC TCA GTA ATA ATA GAG TCT TTA
phe leu gln leu tyr gln lys leu phe lys gln leu his thr met ile ile glu ser ile
val ile ala pro ile thr glu pro ile glu ala leu ser asp asn asn asp OPA phe asp
cys asn cys thr asn asn OPA ser asn arg cys thr leu OPA OCH OCH arg leu phe arg

1081/361
leu
ala
arg ser
CGC TCC
GCG AGG
ala gly
ser
glu

The longest open reading frame in the 1087 nucleotides of the insert, 176 amino acids, starts at nucleotide 247 (CAA CAC ...) in a phase 1 translation. It starts at the first nucleotide and proceeds left to right.

This sequence was obtained as described in Example 17 by reading into the DNA insert and generating sequencing primers. Table 5 below provides the enzyme restriction map of this clone, generated by computer from the sequence itself.

Table 5: Enzyme Restriction Map of DNA from Clone S34



Example 19: DNA Sequence of Clone S19

A partial DNA nucleotide sequence of the *C. parvum* insert of clone S19 was determined as described for clone S34. The DNA sequence obtained and its complement are shown in Table 6 below as are the six amino acid sequences deduced therefrom.

Table 6

DNA and amino acid sequences of clone S19

1/1 31/11

phe arg gln leu met pro asn asn gln leu arg leu ala arg gly gly asn leu cys OCH
ile pro ala ile asn ala lys gln ser ala gln ile ser AMB arg trp lys ser met leu
asn ser gly asn OCH cys gln thr ile ser ser asp AMB leu glu val glu ile tyr val
AAT TCC GGC AAT TAA TGC CAA ACA ATC AGC TCA GAT TAG CTA GAG GTG GAA ATC TAT GTT
TTA AGG CCG TTA ATT ACG GTT TGT TAG TCG AGT CTA ATC GAT CTC CAC CTT TAG ATA CAA
ile gly ala ile leu ala leu cys asp ala OPA ile leu AMB leu his phe asp ile asn
asn arg cys asn ile gly phe leu OPA ser leu asn ala leu pro pro phe arg his OCH
glu pro leu OCH his trp val ile leu glu ser OCH ser ser thr ser ile AMB thr leu

61/21 91/31

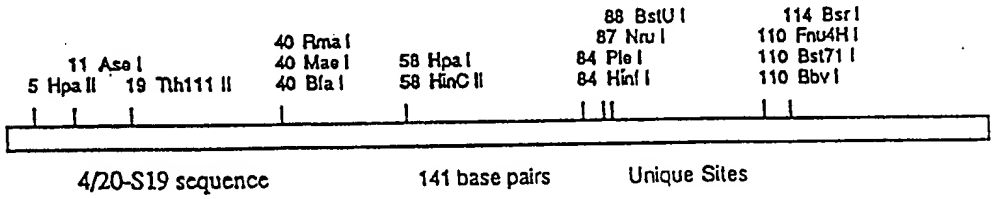
gln val gln glu ile ser gln glu ser arg met leu his OCH thr gln gln pro val pro
thr ser pro gly asp lys pro gly val ala asn val ala leu asn ser ala ala ser ser
asn lys ser arg arg OCH ala arg ser arg glu cys cys ile lys leu ser ser gln phe
AAC AAG TCC AGG AGA TAA GCC AGG AGT CGC GAA TGT TGC ATT AAA CTC AGC AGC CAG TTC
TTG TTC AGG TCC TCT ATT CGG TCC TCA GCG CTT ACA ACG TAA TTT GAG TCG TCG GTC AAG
val leu gly pro ser leu gly pro thr ala phe thr ala asn phe glu ala ala leu glu
cys thr trp ser ile leu trp ser asp arg ile asn cys OCH val OPA cys gly thr gly
leu asp leu leu tyr ala leu leu arg ser his gln met leu ser leu leu trp asn trp

121/41

gln val cys AMB thr val
thr ser val leu asp ser
his lys cys val arg gln tyr
CAC AAG TGT GTT AGA CAG TAT
GTG TTC ACA CAA TCT GTC ATA
val leu thr asn ser leu ile
cys thr his OCH val thr
leu his thr leu cys tyr

This sequence was obtained using universal primers (US Biochemical). Table 7 below provides the enzyme restriction map of this clone generated from the sequence.

Table 7: Enzyme Restriction Map of DNA from Clone S19



The correct reading frame for the 141 nucleotides of the insert remains to be determined. Antibodies elicited by immunization with *C. parvum* that bind to the 68 and 95 kD peptide bands of the *C. parvum* lysate also bind to the S19 polypeptide.

Example 20: DNA Sequence of Clone S7

The DNA nucleotide sequence of the *C. parvum* insert of clone S7 was determined as described for the S34 clone. The DNA sequence obtained and that of its complementary stand are shown in Table 8 below as are the six possible animo acid sequences deduced therefrom.

Table 8

DNA and amino acid sequences of clone S7

1/1 31/11

gly asn tyr pro ser leu leu leu arg asp ile thr glu asp ile AMB val gln asn lys
arg glu leu pro leu ile ile ala ser arg tyr asn OPA gly tyr leu gly thr lys OCH
pro gly ile thr pro his tyr cys phe glu ile OCH leu arg ile phe arg tyr lys ile
CCG GGA ATT ACC CCT CAT TAT TGC TTC GAG ATA TAA CTG AGG ATA TTT AGG TAC AAA ATA
GGC CCT TAA TGG GGA GTA ATA ACG AAG CTC TAT ATT GAC TCC TAT AAA TCC ATG TTT TAT
arg ser asn gly arg met ile ala glu leu tyr leu gln pro tyr lys pro val phe tyr
pro phe OCH gly glu asn asn ser arg ser ile val ser ser ile OCH thr cys phe leu
pro ile val gly OPA OCH gln lys ser ile tyr ser leu ile asn leu tyr leu ile phe

61/21 91/31

leu thr leu asn gln OCH ile gln phe val asn leu ile OCH phe pro val lys OPA asn
ile asp leu glu ser ile asn ser ile cys glu phe asn leu ile ser ser lys val lys
asn OPA pro OPA ile asn lys phe asn leu OPA ile OCH phe asn phe gln OCH ser glu
AAT TGA CCT TGA ATC AAT AAA TTC AAT TTG TGA ATT TAA TTT AAT TTC CAG TAA AGT GAA
TTA ACT GGA ACT TAG TTA TTT AAG TTA AAC ACT TAA ATT AAA TTA AAG GTC ATT TCA CTT
ile ser arg ser asp ile phe glu ile gln ser asn leu lys ile glu leu leu thr phe
asn val lys phe OPA tyr ile OPA asn thr phe lys ile OCH asn gly thr phe his phe
gln gly gln ile leu leu asn leu lys his ile OCH asn leu lys trp tyr leu ser ile

121/41 151/51

ile phe tyr lys ile arg tyr tyr ser ile leu lys tyr thr ile val tyr OPA asn glu
tyr phe leu gln asn ser leu leu phe his phe lys val tyr asn ser leu leu lys OPA
ile phe phe thr lys phe ala ile ile pro phe OCH ser ile gln AMB ser ile glu met
ATA TTT TTT ACA AAA TTC GCT ATT ATT CCA TTT TAA AGT ATA CAA TAG TCT ATT GAA ATG
TAT AAA AAA TGT TTT AAG CGA TAA TAA GGT AAA ATT TCA TAT GTT ATC AGA TAA CTT TAC
tyr lys lys cys phe glu ser asn asn trp lys leu thr tyr leu leu arg asn phe his
ile lys OCH leu ile arg AMB OCH glu met lys phe tyr val ile thr AMB gln phe ser
asn lys val phe asn ala ile ile gly asn OCH leu ile cys tyr asp ile ser ile leu

181/61 211/71

tyr ile ser AMB lys val ser thr lys leu tyr cys OPA ser cys tyr gly arg trp phe
val his AMB leu glu ser AMB his gln thr leu leu leu lys leu leu trp gln met val
ser thr leu val arg lys leu ala pro asn phe thr ala glu ala val met ala asp gly
AGT ACA TTA GTT AGA AAG TTA GCA CCA AAC TTT ACT GCT GAA GCT GTT ATG GCA GAT GGT
TCA TGT AAT CAA TCT TTC AAT CGT GGT TTG AAA TGA CGA CTT CGA CAA TAC CGT CTA CCA
thr cys OCH asn ser leu OCH cys trp val lys ser ser phe ser asn his cys ile thr
tyr met leu OCH phe thr leu val leu ser OCH gln gln leu gln OCH pro leu his asn
val asn thr leu phe asn ala gly phe lys val ala ser ala thr ile ala ser pro glu

Table 8 (cont.)

241/81 271/91

ile gln glu gly leu leu glu arg leu gln arg lys ile arg cys ile val leu leu ser
his ser arg arg ser pro OPA ala thr thr glu glu asn thr leu tyr cys ser ser ile
ser phe lys lys val ser leu ser asp tyr arg gly lys tyr val val leu phe phe tyr
TCA TTC AAG AAG GTC TCC TTG AGC GAC TAC AGA GGA AAA TAC GTT GTA TTG TTC TTC TAT
AGT AAG TTC TTC CAG AGG AAC TCG CTG ATG TCT CCT TTT ATG CAA CAT AAC AAG AAG ATA
OPA glu leu leu asp gly gln ala val val ser ser phe val asn tyr gln glu glu ile
met OPA ser pro arg arg ser arg ser cys leu phe ile arg gln ile thr arg arg asp
asn leu phe thr glu lys leu ser AMB leu pro phe tyr thr thr asn asn lys AMB gly

301/101 331/111

ile lys leu his ile cys met ser ile OPA asn leu ser ile gln ser ser thr lys arg
his OCH thr ser his leu tyr val his leu lys ser AMB his ser ile lys his lys lys
pro leu asn phe thr phe val cys pro ser glu ile leu ala phe asn gln ala gln lys
CCA TTA AAC TTC ACA TTT GTA TGT CCA TCT GAA ATC TTA GCA TTC AAT CAA GCA CAA AAA
GGT AAT TTG AAG TGT AAA CAT ACA GGT AGA CTT TAG AAT CGT AAG TTA GTT CGT GTT TTT
trp OCH val glu cys lys tyr thr trp arg phe asp OCH cys glu ile leu cys leu phe
met leu ser OPA met gln ile asp met gln phe arg leu met OPA asp leu val phe leu
asn phe lys val asn thr his gly asp ser ile lys ala asn leu OPA ala cys phe ser

361/121 391/131

leu OPA glu ile gly ser thr ala pro leu val cys ser val asp ser gln tyr ser his
thr leu arg asn trp glu tyr ser ser ser arg val leu ser OPA phe ser ile leu pro
asp phe glu lys leu gly val gln leu leu ser cys ala gln leu ile leu asn thr pro
GAC TTT GAG AAA TTG GGA GTA CAG CTC CTC TCG TGT GCT CAG TTG ATT CTC AAT ACT CCC
CTG AAA CTC TTT AAC CCT CAT GTC GAG GAG AGC ACA CGA GTC AAC TAA GAG TTA TGA GGG
val lys leu phe gln ser tyr leu glu glu arg thr ser leu gln asn glu ile ser gly
ser gln ser ile pro leu val ala gly arg thr his glu thr ser glu OPA tyr glu trp
lys ser phe asn pro thr cys ser arg glu his ala OPA asn ile arg leu val gly met

421/141 451/151

ala ala trp arg arg thr pro leu glu gln gly gly ile gly pro val asn phe pro leu
cys cys met glu thr tyr ser ser OPA thr arg trp asn trp thr ser gln phe pro thr
met leu his gly asp val leu leu leu asn lys val glu leu asp gln ser ile ser his
ATG CTG CAT GGA GAC GTA CTC CTC TTG AAC AAG GTG GAA TTG GAC CAG TCA ATT TCC CAC
TAC GAC GTA CCT CTG CAT GAG GAG AAC TTG TTC CAC CTT AAC CTG GTC AGT TAA AGG GTG
his gln met ser val tyr glu glu gln val leu his phe gln val leu OPA asn gly val
ala ala his leu arg val gly arg ser cys pro pro ile pro gly thr leu lys gly ser
ser cys pro ser thr ser arg lys phe leu thr ser asn ser trp asp ile glu trp lys

Table 8 (cont.)

481/161 511/171
ile ser asp ser ser his ser ile ser lys asn tyr gly val leu ser arg gly arg arg
tyr leu OPA leu ile ser phe asn AMB gln glu leu trp cys thr phe ser arg lys lys
leu ser leu thr his leu ile gln leu ala arg thr met val tyr phe leu glu glu glu
TTA TCT CTG ACT CAT CTC ATT CAA TTA GCA AGA ACT ATG GTG TAC TTT CTC GAG GAA GAA
AAT AGA GAC TGA GTA GAG TAA GTT AAT CGT TCT TGA TAC CAC ATG AAA GAG CTC CTT CTT
OCH arg gln ser met glu asn leu OCH cys ser ser his his val lys glu leu phe phe
ile glu ser glu asp OPA glu ile leu leu phe AMB pro thr ser glu arg pro leu leu
asp arg val OPA arg met OPA asn ala leu val ile thr tyr lys arg ser ser ser pro

541/181 571/191
tyr cys ser gln arg phe ile his his OPA gln gly gly ser arg cys ser phe OPA ser
val leu leu ser glu val tyr ser ser leu thr arg arg val ala leu phe val leu lys
gly ile ala leu arg gly leu phe ile ile asp lys glu gly arg val val arg ser glu
GGT ATT GCT CTC AGA GGT TTA TTC ATC ATT GAC AAG GAG GGT CGC GTT GTT CGT TCT GAA
CCA TAA CGA GAG TCT CCA AAT AAG TAG TAA CTG TTC CTC CCA GCG CAA CAA GCA AGA CTT
thr asn ser glu ser thr OCH glu asp asn val leu leu thr ala asn asn thr arg phe
tyr gln glu OPA leu asn ile OPA OPA gln cys pro pro asp arg gln glu asn gln leu
ile ala arg leu pro lys asn met met ser leu ser pro arg thr thr arg glu ser thr

601/201 631/211
asn leu OPA leu thr ile arg lys ile ser arg arg asn ser thr cys tyr OPA cys thr
OCH ser met thr tyr his AMB glu asp gln ser lys lys leu tyr val leu leu met his
val ile tyr asp leu pro leu gly arg ser val glu glu thr leu arg val ile asp ala
GTA ATC TAT GAC TTA CCA TTA GGA AGA TCA GTC GAA GAA ACT CTA CGT GTT ATT GAT GCA
CAT TAG ATA CTG AAT GGT AAT CCT TCT AGT CAG CTT CTT TGA GAT GCA CAA TAA CTA CGT
tyr asp ile val OCH trp OCH ser ser OPA asp phe phe ser AMB thr asn asn ile cys
leu arg his ser val met leu phe ile leu arg leu phe glu val his OCH gln his val
ile AMB ser lys gly asn pro leu asp thr ser ser val arg arg thr ile ser ala ser

661/221 691/231
ser ile his OPA asn leu trp OPA ser leu pro ser lys leu glu glu gly pro lys arg
phe asn ser leu lys pro met val lys phe ala gln gln thr gly arg arg ala lys lys
leu gln phe thr glu thr tyr gly glu val cys pro ala asn trp lys lys gly gln lys
CTT CAA TTC ACT GAA ACC TAT GGT GAA GTT TGC CCA GCA AAC TGG AAG AAG GGC CAA AAA
GAA GTT AAG TGA CTT TGG ATA CCA CTT CAA ACG GGT CGT TTG ACC TTC TTC CCG GTT TTT
lys leu glu ser phe gly ile thr phe asn ala trp cys val pro leu leu ala leu phe
glu ile OPA gln phe arg his his leu lys gly leu leu ser ser ser pro gly phe leu
OPA asn val ser val AMB pro ser thr gln gly ala phe gln phe phe pro trp phe pro

Table 8 (cont.)

721/241 751/251
asn val ser tyr ser OPA arg cys phe gln leu ser OCH gly leu ile leu glu OPA phe
glu cys gln leu leu met lys val phe pro val ile leu arg thr his phe arg met ile
gly met ser ala thr his glu gly val ser ser tyr leu lys asp ser phe AMB asn asp
GGA ATG TCA GCT ACT CAT GAA GGT GTT TCC AGT TAT CTT AAG GAC TCA TTT TAG AAT GAT
CCT TAC AGT CGA TGA GTA CTT CCA CAA AGG TCA ATA GAA TTC CTG AGT AAA ATC TTA CTA
ser his OPA ser ser met phe thr asn gly thr ile lys leu val OPA lys leu ile ile
phe thr leu AMB glu his leu his lys trp asn asp OCH pro ser met lys ser his asn
ile asp ala val OPA ser pro thr glu leu OCH arg leu ser glu asn OCH phe ser lys

781/261 811/271
asn phe ser asn glu pro asn phe phe leu ile OPA leu phe leu cys ser tyr lys ser
OCH phe phe lys OPA thr lys phe phe phe asn leu thr phe phe met AMB leu OCH ile
leu ile phe gln met asn gln ile phe phe OCH ser asp phe phe tyr val val ile asn
TTA ATT TTT CAA ATG AAC CAA ATT TTT TTT TAA TCT GAC TTT TTT TAT GTA GTT ATA AAT
AAT TAA AAA GTT TAC TTG GTT TAA AAA AAA ATT AGA CTG AAA AAA ATA CAT CAA TAT TTA
OCH asn lys leu his val leu asn lys lys leu arg val lys lys ile tyr asn tyr ile
leu lys glu phe ser gly phe lys lys lys ile gln ser lys lys his leu OCH leu asp
ile lys OPA ile phe trp ile lys lys OCH asp ser lys lys OCH thr thr ile phe OPA

841/281 871/291
asp ala asn glu tyr arg arg leu his ile AMB ile leu cys gly asp AMB ile val glu
arg cys lys OPA val ser ser ser pro his leu asp pro leu trp arg leu asp cys gly
gln met gln met ser ile val val ser thr ser arg ser ser val ala thr arg leu trp
CAG ATG CAA ATG AGT ATC GTC GTC TCC ACA TCT AGA TCC TCT GTG GCG ACT AGA TTG TGG
GTC TAC GTT TAC TCA TAG CAG CAG AGG TGT AGA TCT AGG AGA CAC CGC TGA TCT AAC ACC
leu his leu his thr asp asp asp gly cys arg ser gly arg his arg ser ser gln pro
ser ala phe ser tyr arg arg arg trp met AMB ile arg gln pro ser AMB ile thr ser
ile cys ile leu ile thr thr glu val asp leu asp glu thr ala val leu asn his phe

901/301 931/311
AMB val gln ile asn pro gly val val asn val val leu asn phe cys asn leu ser phe
ile gly ala asn lys pro trp ser cys OCH cys ser val lys phe leu OCH phe ile phe
asn arg cys lys OCH thr leu glu leu leu met AMB cys OCH ile phe val ile tyr leu
AAT AGG TGC AAA TAA ACC CTG GAG TTG TTA ATG TAG TGT TAA ATT TTT GTA ATT TAT CTT
TTA TCC ACG TTT ATT TGG GAC CTC AAC AAT TAC ATC ACA ATT TAA AAA CAT TAA ATA GAA
ile pro ala phe leu gly gln leu gln OCH his leu thr leu asn lys tyr asn ile lys
tyr thr cys ile phe gly pro thr thr leu thr thr asn phe lys gln leu lys asp lys
leu his leu tyr val arg ser asn asn ile tyr his OCH ile lys thr ile OCH arg lys

Table 8 (cont.)

961/321 991/331

phe phe leu leu thr tyr leu ser trp cys AMB gln ser ser ile arg asn his tyr ser
phe phe leu ile asn leu pro phe leu val leu ala val phe tyr AMB lys ser leu leu
phe phe ser tyr OCH leu thr phe leu gly val ser ser leu leu leu glu ile ile thr
TTT TTT TCT TAT TAA CTT ACC TTT CTT GGT GTT AGC AGT CTT CTA TTA GAA ATC ATT ACT
AAA AAA AGA ATA ATT GAA TGG AAA GAA CCA CAA TCG TCA GAA GAT AAT CTT TAG TAA TGA
lys lys arg ile leu lys gly lys lys thr asn ala thr lys AMB OCH phe asp asn ser
lys lys lys asn val OCH arg glu gln his OCH cys asp glu ile leu phe OPA OCH glu
lys glu OCH OCH ser val lys arg pro thr leu leu arg arg asn ser ile met val arg

1021/341 1051/351

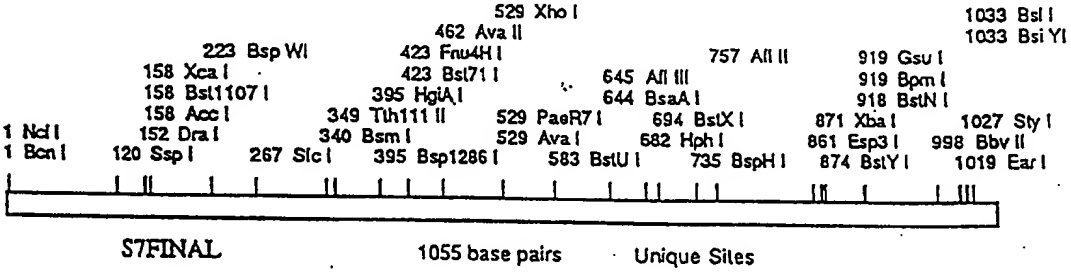
ser ser lys ala gly arg arg ser pro OPA
phe phe gln gly arg lys lys val ser leu
leu leu pro arg pro glu glu gly leu leu glu
CTT CTT CCA AGG CCG GAA GAA GGT CTC CTT GAG
GAA GAA GGT TCC GGC CTT CTT CCA GAG GAA CTC
lys lys trp pro arg phe phe thr glu lys leu
glu glu leu ala pro leu leu asp gly gln
arg gly leu gly ser ser pro arg arg ser

The longest open reading frame comprises 201 amino acids of the 1053 nucleotides of the insert. It starts at nucleotide 169 (TCT ATT ...) in a phase 1 translation (starting at the first nucleotide and preceding left to right).

Extensive homology was observed between this sequence and the 20 kD protein of *Clostridium pasteruanium*, the 29 kD surface protein of *Entamoeba histolytica*, a *Helicobacter pylori* protein, and the 22 kD subunit of the alkyl hydroperoxide reductase of *Salmonella typhimurium*, having GenBank accession numbers _____ (C. past.), M75858 (E. hist.), M55507 (H. pylori) and M05478 (S. typh.), respectively.

This sequence was obtained by the successive generation of sequencing primers as described for clone S34. Table 9 below provides the enzyme restriction map for this clone derived from the sequence.

Table 9: Enzyme Restriction Map of DNA from Clone S7



Antibodies elicited by *C. parvum* immunization that bind to the 23 kD apparent molecular weight peptide also bind to the S7 polypeptide.

The subgroup of antibodies binding to the 23 kD group of polypeptides show substantially no binding to other groups.

Example 21: DNA Sequence of Clone S24.

The DNA nucleotide sequence of the *C. parvum* insert of clone S24 was determined as described for the S34 clone. A partial DNA sequence obtained and its complementary strand are shown in Table 10 below as are the six possible acid sequences deduced therefrom.

Table 10

DNA and amino acid sequences of clone S24

1/1 31/11

thr leu asn gln OCH ile gln phe val asn leu ile OCH phe pro val lys OPA asn ile
asp leu glu ser ile asn ser ile cys glu phe asn leu ile ser ser lys val lys tyr
gly pro OPA ile asn lys phe asn leu OPA ile OCH phe asn phe gln OCH ser glu ile
GGA CCT TGA ATC AAT AAA TTC AAT TTG TGA ATT TAA TTT AAT TTC CAG TAA AGT GAA ATA
CCT GGA ACT TAG TTA TTT AAG TTA AAC ACT TAA ATT AAA TTA AAG GTC ATT TCA CTT TAT
ser arg ser asp ile phe glu ile gln ser asn leu lys ile glu leu leu thr phe tyr
val lys phe OPA tyr ile OPA asn thr phe lys ile OCH asn gly thr phe his phe ile
gly gln ile leu leu asn leu lys his ile OCH asn leu lys trp tyr leu ser ile asn

61/21 91/31

phe tyr lys ile gln tyr tyr ser ile leu lys tyr thr ile val tyr OPA asn glu tyr
phe leu gln asn ser val leu phe his phe lys val tyr asn ser leu leu lys OPA val
phe phe thr lys phe ser ile ile pro phe OCH ser ile gln AMB ser ile glu met ser
TTT TTT ACA AAA TTC AGT ATT ATT CCA TTT TAA AGT ATA CAA TAG TCT ATT GAA ATG AGT
AAA AAA TGT TTT AAG TCA TAA TAA GGT AAA ATT TCA TAT GTT ATC AGA TAA CTT TAC TCA
lys lys cys phe glu thr asn asn trp lys leu thr tyr leu leu arg asn phe his thr
lys OCH leu ile OPA tyr OCH glu met lys phe tyr val ile thr AMB gln phe ser tyr
lys val phe asn leu ile ile gly asn OCH leu ile cys tyr asp ile ser ile leu val

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ile ser AMB lys val ser thr lys leu tyr cys OPA ala val met gln met val his ser
his AMB leu glu ser AMB his gln thr leu leu leu ser cys tyr ala asp gly ser phe
thr leu val arg lys leu ala pro asn phe thr ala glu leu leu cys arg trp phe ile
ACA TTA GTT AGA AAG TTA GCA CCA AAC TTT ACT GCT GAG CTG TTA TGC AGA TGG TTC ATT
TGT AAT CAA TCT TTC AAT CGT GGT TTG AAA TGA CGA CTC GAC AAT ACG TCT ACC AAG TAA
cys OCH asn ser leu OCH cys trp val lys ser ser leu gln OCH ala ser pro glu asn
met leu OCH phe thr leu val leu ser OCH gln gln ala thr ile cys ile thr OPA glu
asn thr leu phe asn ala gly phe lys val ala ser ser asn his leu his asn met OPA

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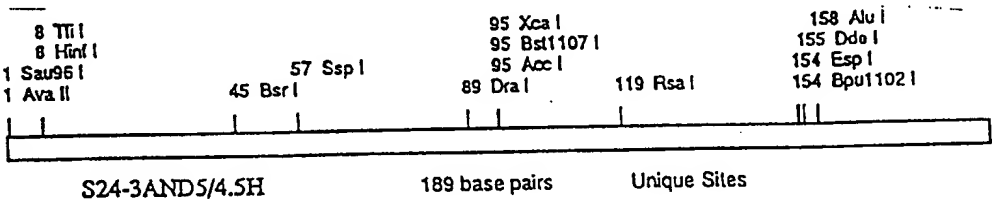
glu val
arg gly
gln arg ser
CAG AGG TCT
GTC TCC AGA
leu pro arg
ser thr
leu asp

There is no obvious candidate for the correct reading frame in the 189 nucleotides of the insert. This sequence was obtained by using the universal primers obtained from US Biochemical. Table 11 below provides the enzyme restriction map of this clone as generated from then sequence.

Table 11:Enzyme Restriction Map of DNA for Clone S24



Antibodies raised against *C.parvum* that bind to the 15 and 35 kD apparent molecular weight peptides also bind to the S24 polypeptide. This subgroup of antibodies shows substantially no binding activity with respect to the polypeptides of the other antigenic groups.

Example 22: Agents Suitable for Passive Immunotherapy

The polypeptides of the invention bind to antibodies also specifically binding to epitopes of *C. parvum*. These *C.parvum* epitopes are also recognized by B and T cells. The polypeptides mentioned above are produced in large amounts by reinserting the *C. parvum* DNA from the different clones obtained in the Examples above into an expression vector such as pGEX, pET-9d, or baculovirus. The thus constructed hybrid vector is used to transfect a host. The host cells carrying the hybrid vector are then grown in a nutrient medium to allow the production of the gene product.

pGEX (Pharmacia)(Smith, Gene 67:31 (1988)) or pET-9d (Novagen)/pRSET T7 (Invitrogen) utilize the T7 RNA

polymerase and the T7 promoter (Studier, F. W., Meth. Enzymol. 185:60 (1990)) and hosts derived from *E. coli*. The vector sequences may be easily eliminated following protein expression so that the subsequent immunogenic protein contains only *Cryptosporidium* sequences. These expression systems are commercially available and their use is standard in the art.

Recombinant baculovirus is a simple vehicle for the expression of large quantities of protein from eukaryotic or prokaryotic gene origin. The genes are expressed under the control of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedral promoter contained in transfer vectors used to infect *Spodoptera frugiperda* (Sf9 or Sf21) insect cells. A number of transfer vectors are available for the production of protein from both full length and partial cDNA and genomic clones. Fused or non-fused protein products, depending on the vector used, constitute up to 50% of the total protein produced in infected cells. The thus obtained recombinant proteins are frequently immunologically and functionally similar to the corresponding endogenous proteins. Proteins with signal peptides may be secreted into the media while those without secretion signals will aggregate in the cells or be localized at the membrane. Baculovirus expression systems are commercially available (Invitrogen), (Smith, D., and Johnson, K. , (1988), supra; Studier, F. W., et al. (1990), supra).

The thus obtained polypeptide is purified by methods known in the art, and the degree of purification varies with the use of the polypeptide. For use in eliciting polyclonal antibodies, the degree of purity may not need to be high. However, in some cases impurities may cause adverse reactions and the degree of purity must be higher.

When used to passively immunize *Cryptosporidium* infected animals, the polypeptide is first combined with appropriate adjuvants and used for the immunization of cows or other donor animals to produce antibodies which may be administered to patients with *cryptosporidiosis* infection,

particularly to AIDS patients and other immunocompromised hosts, including animals.

Example 23: Agents Suitable for Active Immunotherapy

Polypeptides comprising epitopes of *C.parvum* recognized by B and/or T cells are produced in large amounts by recloning as described in Example 22 above.

The polypeptide thus obtained is purified as described above (e.g. Smith, D., and Johnson, K., (1988), supra).

The degree of purification varies with the use of the polypeptides. For use in eliciting polyclonal antibodies, the degree of purity may be lower than for other applications. For the preparation of a pharmaceutical composition, however, the degree of purity must be high, as is known in the art.

When in a therapeutic composition, the polypeptide is combined with appropriate adjuvants and used for the immunization of immuno-competent patients who are at risk for cryptosporidiosis either at the time of immunization of in the future. This group includes, but is not restricted to, HIV positive individuals who are still able to respond to vaccination, animal workers, health care workers, day care center children and their caretakers, and children in the developing world.

Example 24: Agents Suitable for Immunodiagnostic Use

Polypeptides comprising epitopes of *C. parvum* that are recognized by intact B and/or T cells are produced in large amounts as described above, purified and used to detect or characterize *anti-C. parvum* antibody in the body substances of populations at risk of prior or current cryptosporidial infection. In addition, antibodies to such polypeptides are obtained by immunizing animals, such as rabbits or goats, with the polypeptide plus adjuvant as described. Typical intramuscular immunization schedules are as follows.

1) Polypeptide plus equal volume complete Freunds adjuvant at the beginning.
2) Polypeptide plus equal volume incomplete Freunds adjuvant at week 2.

3) Polypeptide plus equal volume incomplete Freunds adjuvant at week 4.

These antibodies are used to detect *Cryptosporidium* antigens in body substances, for example, stools of populations at risk of cryptosporidial infection by, e.g., collecting stool samples (Manual of Clinical Microbiology (1986), supra), mixing with Streather's solution 1:4, and incubating with antibody followed by addition of a fluorescein conjugated second antibody as described. However, colorimetric labels which do not require special microscope equipment are suitable.

Example 25: Anti-*C. parvum* Antibodies Eluted from Western Blot

For SDS-PAGE, $2x10^9$ oocysts were lysed by 5 cycles of freeze-thawing in 1% Triton Buffer (150 mM Na Cl; 100 mM EDTA; and 1% Triton X-100), in the presence of protease inhibitors (100 μM E64, chymotrypsin, pepstatin, and leupeptin; and 1.6 mM PMSF), and boiled in Sample Buffer (SB). Proteins were electrophoresed in 5-15% gradient gels (Laemmli, U. K., "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4", Nature 227:680 (1971)) and blotted onto nitrocellulose at 0.7 amp. for 8 hrs. (Petersen, C., et al., "Characterization of an Mr>900,000 *Cryptosporidium parvum* Sporozoites Glycoprotein Recognized by Hyperimmune Bovine Colostral Immunoglobulin", Inf. & Immun. 60(12):5132 (1992)). Western blots were incubated with HBC Ig (lot # 40529) (dil 1/500) in 20 ml PBS for 3 hrs. at 4 °C, rinsed 3 times with phosphate buffered saline (PBS), and antibodies eluted with 10 ml of glycine buffer (pH 2.6) for 3 min., followed by addition of a 1/10 volume of 2M Tris buffer, pH 8 (Tilley, M., et al., "*Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) Oocyst and Sporozoite Antigens Recognized by Bovine Colostral Antibodies", Inf. Imm. 58:2966 (1990)). Eluted antibodies were filter sterilized and concentrated to a final volume of 1 ml in a Centriprep 10 concentrator (Amicon, MA).

Example 26: *In vitro* **Inhibition Assay**

The *in vitro* cell culture system of Gut et al. was modified as follows to quantify the effect of antibody on the infection of epithelial cells by *C. parvum* (Gut J., et al., "*Cryptosporidium parvum: In vitro* Cultivation in Madin-Darby Canine Kidney Cells" J. Protozool. 386:72 (1991)). MDCK cells were maintained in RPMI-1640 with the addition of 5% heat inactivated fetal calf serum (FCS). Two ml aliquots containing $2x10^5$ MDCK cells/ml were seeded in 8 well tissue culture plates and allowed to attach to 20 mm square cover glasses for 24 h at 37 °C in a 5% CO_2: 95% air atmosphere.

The cells were then rinsed in RPMI without FCS for 30 minutes, exposed to 2 x 10^6purified oocysts resuspended in RPMI medium containing HBC Ig (lot# 40529) (1000; 500; 200; 100; and 50 μg/ml IgG), SHAM-HBC Ig (lot # 41038) (1500; 250; 150; and 75 μg/ml IgG), eluted antibodies (50-100 μg/ml IgG), or 5% FCS (300-500 μg/ml IgG). In some experiments, controls were also conducted consisting of MDCK cells infected with *C. parvum* oocysts resuspended in RPMI medium with the addition of glycine buffer at the same concentration utilized for the cultures treated with eluted antibody as described above.

The cultures were incubated for 2 hrs. at 37 °C, rinsed 4 times with RPMI to remove extracellular sporozoites and unexcysted oocysts, and reincubated for an additional 21 hrs. period in the presence of the respective antibody reagents described above. Monolayers were subsequently fixed in 3.7 % formaldehyde in PBS, rinsed, and stained in PBS with the addition of 1 μM Hoescht 33258 dye (Sigma) for 1 hr. at 37 °C (Melamed, M. R., and L. A. Kamentsky, "Automated Cytology", Exp. Pathol. 14:205 (1975)).

The number of intracellular parasites/200-400 cells was quantified in 3 independent experiments in coded slides (n= 3-4) by fluorescence microscopy. Differences in the mean number of intracellular parasites/cell were statistically analyzed.

The data were expressed as the E/C ratio (± SEM), where E was the mean number of intracellular parasites in the treated culture, and C the mean number of intracellular parasites in the untreated or SHAM-HBC Ig treated controls (Crane, M. S. J., and J. C. McGaley, "*Eimeria tenella*: Inhibition of Host Cell Invasion by Phospholipase Treatment of Sporozoites", Exp. Parasitol. 72:219 (1991); Doyle, P. S., et al., "*Trypanosoma cruzi*: Quantification and Analysis of the Infectivity of Cloned Stocks", J. Protozool. 31:2806 (1984)).

Example 27: Western Blots

To identify the molecular targets of protective antibody, total *C. parvum* sporozoite and sporozoite/oocyst proteins were boiled in sample buffer (SB), resolved in 5-15% gradient gels by SDS-PAGE and Western blotted with HBC Ig. In addition, sporozoite/oocyst proteins solubilized in Triton-X 100 were immunoprecipitated with HBC Ig at dilutions 1/1,000; 1/5,000; 1/10,000; 1/50,000 and 1/100,000. Controls were *C. parvum* proteins immunoprecipitated under the same conditions but with SHAM-HBC Ig at dilutions 1/1,000 to 1/10,000. Immunoprecipitates were also resolved by SDS-PAGE and Western blotted. Western blots of HBC Ig immunoprecipitates were developed with HBC Ig (dil 1/1,000) and SHAM immunoprecipitates were developed with SHAM-HBC Ig (dil 1/1,000). After incubation with 10 μCi [^{125} I]-Protein G for 1 hr. at room temperature, blots were dried and exposed for autoradiography.

Example 28: Discussion of HBC Ig Inhibition Results

The *in vitro* assay described in Example 26 above was used to determine inhibition of *Cryptosporidium* invasion and/or intracellular development as a function of HBC Ig titer. HBC Ig, at concentrations ranging from 100-1,000 μg/ml IgG, resulted in a significant reduction in the mean number of intracellular parasites/host cell of up to 61% relative to FCS controls as can be seen in Figure 1

($p<0.01$), while no inhibition was observed at lower HBC Ig ($\leq$ 50 μg/ml of IgG) concentrations. A second control, SHAM-HBC Ig, was found not to significantly inhibit *C. parvum* infectivity relative to 5% FCS controls, although this reagent does contain some anti-*Cryptosporidium* activity by ELISA as shown in Figure 2. In addition, HBC Ig (100-1,000 μg/ml IgG) significantly inhibited *Cryptosporidium* invasion and/or development when compared to SHAM-HBC Ig controls by 45-55%. Specific anti-*C. parvum* antibodies were affinity purified from Western blot (about 50-100 μg IgG/ml). This eluted antibody also inhibited *Cryptosporidium* invasion/development relative to SHAM-HBC Ig controls, and controls with the addition of glycine buffer or FCS as shown in Figure 3 ($p<0.01$).

Example 29: Discussion of Immunoprecipitation and Western Blots using Antibodies

When total oocyst/sporozoite proteins were immunoprecipitated with HBC Ig at different concentrations and blotted, two sporozoite molecules of >900 kD and about 250 kD were the major antigenic targets identified by protective colostrum (Figure 4, lanes 1-4) but not by SHAM-HBC Ig (lanes 5-7) at all the Ab concentrations assayed. Most of the *C. parvum* antigenic proteins recognized by HBC Ig are expressed by sporozoites as evidenced by the comparison of total sporozoite and oocyst/sporozoite proteins recognized by HBC Ig in Western blots (data not shown). *Cryptosporidium* sporozoites were also radioiodinated to identify antigens localized to their surface. Twenty-two surface iodinatable sporozoite proteins were resolved by SDS-PAGE (Figure 5, lane 1). Protective anti-*Cryptosporidium* antibodies (lot #40529) immunoprecipitated most of these surface labeled sporozoite proteins. The number of radiolabeled immunoprecipitated proteins resolved by gel electrophoresis differed for membranes solubilized with SDS or with Triton X-100. Nineteen labeled sporozoite surface proteins extracted with SDS were specifically immunoprecipitated by HBC Ig (Figure

5, lane 2) including a >900 kD and about 250 kD molecules (Figure 5B). Only thirteen radioiodinated 1% Triton X-100 soluble proteins were immunoprecipitated by protective HBC immunoglobulins (Figure 5, lane 3) including the >900 kD species (Figure 5B). No radioiodinated molecules were observed in TCA precipitated controls of the soluble cytoplasmic fraction (data not shown) indicating that only membrane proteins were iodinated by this methodology.

Example 30: Production of Antibody to Fusion Protein

A recombinant λgt 11 bacteriophage carrying DNA sequences encoding *C. parvum* polypeptides, such as the S2, S19 and S34 proteins, was used to infect E. coli Y1089 as described in Promega Protocols and Applications, 2nd ed., p. 228 (Promega Corp, Madison, WI). Lysogens grown on LB agar containing ampicillin and tetracycline at 32°C were selected when clones exhibited confluent growth at 32°C but spotty growth at the lytic temperature of 42°C. Single colonies of the lysogens were chosen and grown in LB broth until the A_{600}=0.5. The temperature was then raised to 42°C for 20 min., IPTG was added to 6 mM to induce fusion protein synthesis, and the cultures were grown for an additional 2.5 hrs. at 42°C. The bacteria were harvested by centrifugation, freeze-thawed 3 times, lysozyme treated and vortexed to reduce the viscosity due to released nucleic acids.

The fusion proteins were purified from the bacterial lysates by affinity chromatography on anti-β galactosidase-agarose (Protosorb LacZ, Promega Corp.) according to manufacturers instructions with elution of the fusion proteins using 100 mM sodium carbonate, pH 10.8. S2, S19 and S34 derived fusion proteins were successfully purified using this methodology.

Two CD-1 adult mice were separately immunized intraperitoneally with about 1 μg fusion protein from each clone at 2 to 4 week intervals using the Ribi Adjuvant System (Ribi Immunochem Research, Hamilton, MT) Freund's Complete Adjuvant. The mice receiving the S34 antigen were

boosted with 5 to 10 μg fusion protein excised from SDS polyacrylamide gel, minced and extruded through a hypodermic needle, which was administered i.p. After 7 total immunizations, the production of ascites was induced by the i.p. inoculation of animals with Freund's Complete Adjuvant. The resulting ascites were used to probe western blotted *Cryptosporidium parvum* oocyst lysates. As expected, the anti-S2 ascites recognized about 45 kD band, the anti-S19 ascites recognized the about 68 kD band, and the anti-S34 ascites recognized a band migrating at the stacking gel interface (>250kD).

Example 31: *In Vitro* Inhibition of *C. parvum* Infectivity by anti-S19, S34, and S2 *Cryptosporidium* Protein Antibodies

An *in vitro* cell culture system was utilized to quantify the effect of antibody on the infection of epithelial cells by *C. parvum* (Doyle, P., et al., "Anti-*Cryptosporidium parvum* Antibodies Inhibit Infectivity *In Vitro* and *In Vivo*", Inf. Immun. (1993), in press). Briefly, MDCK cells were maintained in RPMI-1640 medium with the addition of 5% heat inactivated fetal calf serum (FCS). Two ml aliquots containing $2x10^5$ MDCK cells/ml were seeded in 8 well tissue culture plates and allowed to attach to 20 mm square cover glasses for 24 hrs. at 37°C in a 5% CO_2: 95% air atmosphere.

The cells were then rinsed in RPMI medium without FCS for 30 min., exposed to $2x10^6$ purified oocysts resuspended in RPMI medium containing the antibodies, i.e., anti-S19 ascites; anti-S34 ascites; anti-S2 ascites; hyperimmune bovine colostrum immunoglobulin (HBC Ig); anti-GP900 mouse ascites raised against the denatured purified glycoprotein (anti-GP900 ascites); a SHAM hyperimmune bovine colostrum (SHAM-HBC Ig) control; and a 5% heat inactivated fetal bovine serum (FCS) control.

The cultures were incubated for 2 hrs. at 37°C, rinsed 4 times with RPMI to remove extracellular sporozoites and unexcysted oocysts, and reincubated for an additional 21

hrs. period in the presence of the respective antibody reagents as described above. Monolayers were subsequently fixed in 3.7% formaldehyde in PBS, rinsed, and stained in PBS with the addition of 1 μM Hoescht 33258 dye (Sigma) for 1 hr. at 37°C. The number of intracellular parasites/200-400 cells was quantified in coded slides (n=2) by fluorescence microscopy. Differences in the mean number of intracellular parasites/cell were statistically analyzed. The results were expressed as the mean number of intracellular parasites per cell (± SD) in the treated cultures and SHAM-HBC Ig and FCS controls and are shown in Figure 6.

The highest degree of inhibition of *C. parvum* infectivity was shown by the anti-S19 protein antibodies. The anti-S34 and anti-S2 protein antibodies inhibited about 2/3 of the infectivity displayed by the control. The HBC Ig and anti-GP900 protein antibodies showed some inhibitory effect but less so than the previous samples.

Example 32: Inhibition of *C. parvum* Sporozoite Infectivity

The following results confirm the findings of Example 31 above.

The ascites were tested for their ability to neutralize *Cryptosporidium* sporozoite infectivity using a modification of the *Cryptosporidium* invasion assay described by Tilley et al (Tilley et al., Infec. Immun. 59:1002-1007 1991). Briefly, MDBK cells were seeded into 8 well plastic chamber slides (Labtek, Nunc, Napersville, IL) at a concentration of 4 x 10^4 cells/well in Dylbecca's Modified Eagle medium (DME)/2% Fetal Bovine Serum (FBS) and grown for 3 days to greater than 90% confuency. Purified *C. parvum* oocysts from a virulent calf isolate, recently passaged in a newborn calf, were excysted by exposure to 0.75% (w/v) sodium taurocholate for 1 hour at 37°C. Sporozoites were purified by passage through a 2 μm polycarbonate filter (Nuclepore, Costar Inc.) in a 25 mm Swinnex filter housing (Millipore Corp., Bedford MA). 1.25 x 10^6 sporozoites in DME/2% FBS were added to the reaction

mixtures containing test or control antibodies at a final dilution of 1:10 in 0.5 ml DME/2% FBS. After 1 hr. of incubation at room temperature, 0.4 ml of each mixture were separately applied to cell monolayers and incubated for 24 hrs. at 37°C in 5% CO_2.

The monolayers were washed with PBS and processed for immunofluorescence as follows. The cells were fixed with 10% formalin for 15 min. at room temperature and permeabilized with methanol for 10 min. After washing the monolayer with PBS, Bovine anti-*Cryptosporidium* IgG was added at a dilution of about 1 mg/ml IgG in PBS/ 1% Normal Goat Serum (NGS) for 1hr. at room temperature. The cells were then washed 3 times for 5 min. each time with PBS and FITC goat-anti-bovine IgG (Kirkegaard and Perry Labs, Gaithersburg, MD) was applied at 1:100 in PBS/1%NGS. After 1 hr. at room temperature, the cells were washed with PBS and mounted in 4% n-propyl gallate/80% glycerol for microscopy. Developing cryptosporidial forms were enumerated in 10 fields at 1000 x nominal magnification. The results obtained for three different clones are shown in the following Table 12.

Table 12: Inhibition of *C. parvum* Sporozoite Infectivity

Sample	Dilution	% Inhibition of C. parvum Sporozoite Infectivity
Control mouse sera	1:10	38.75 ± 18
Anti-S2 ascites	1:10	43.75 ± 47
Anti-S19 ascites	1:10	100.00 ± 0
Anti-S34 ascites	1:10	80.00 ± 31

These results clearly indicate an inhibitory effect by anti-S19 and anti-S34 antibodies with respect to the control.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

WHAT IS CLAIMED AS NOVEL AND DESIRED TO BE PATENTED BY LETTERS PATENT OF THE UNITED STATES IS:

1. A biologically pure polypeptide, comprising

a biologically pure, isolated peptide capable of specifically binding to anti-*Cryptosporidium* antibodies.

2. The polypeptide of claim 1, wherein the peptide is selected from the group consisting of peptides capable of binding to a subgroup of *C. parvum* antibodies that selectively bind

a greater than 900 kD app. MW peptide from *C. parvum*;

a peptide selected from the group consisting of the 68 and 95 kD app. MW peptides from *C. parvum*;

a 45 kD app. MW peptide from *C. parvum*;

a 23 kD app. MW peptide from *C. parvum*; and

a peptide selected from the group consisting of the 15 and 32 to 35 kD app. MW peptides from *C. parvum*.

3. The polypeptide of claim 2, wherein

the peptide comprises a glycopeptide of app. MW greater than 900 kD.

4. The polypeptide of claim 3, wherein

the peptide comprises a peptide selected from the group consisting of the amino acid sequences of Table 4.

5. The polypeptide of claim 2, wherein

the peptide has an app. MW selected from the group consisting of 68 kD and 95 kD.

6. The polypeptide of claim 5, wherein the peptide comprises a peptide selected from the group consisting of the amino acid sequences of Table 6.

7. The polypeptide of claim 2, wherein the peptide has an app. MW of 45 kD.

8. The polypeptide of claim 2, wherein the peptide has an app. MW of 23 kD.

9. The polypeptide of claim 8, wherein the peptide comprises a peptide selected from the group consisting of the amino acid sequences of Table 8.

10. The polypeptide of claim 2, wherein the peptide has an app. MW selected from the group consisting of 15 and 32 to 35 kD.

11. The polypeptide of claim 10, wherein the peptide comprises a peptide selected from the group consisting of the amino acid sequences of Table 10.

12. A composition, comprising the polypeptide of Claim 1; and a carrier.

13. A biologically pure DNA segment encoding the polypeptide of claim 1.

14. The DNA segment of claim 13, comprising

a DNA fragment selected from the group consisting of the DNA sequences of Tables 4, 6, 8, and 10, and complementary and degenerate sequences thereof encoding the polypeptide.

15. The DNA segment of claim 13, further comprising

a second unrelated DNA segment operably coupled to the DNA fragment encoding the polypeptide.

16. An RNA segment, encoding the polypeptide of claim 1.

17. The RNA segment of claim 16, comprising

an RNA fragment selected from the group consisting of polydeoxyribonucleotide segments corresponding to the DNA segments of Tables 4, 6, 8 and 10, and complementary and degenerate sequences thereof encoding the polypeptide.

18. The RNA segment of claim 16, further comprising

a second unrelated RNA segment operably coupled to the RNA segment encoding the polypeptide.

19. A fusion protein, comprising

the polypeptide of claim 1; and

a second unrelated polypeptide operably coupled to the polypeptide.

20. A hybrid vector, comprising

a vector capable of replication and expression of a DNA segment operably coupled thereto; and

the DNA segment of claim 13 operably coupled thereto, wherein when the hybrid vector is placed in an appropriate host, it can express the polypeptide encoded by the DNA segment.

21. A host, comprising

the hybrid vector of claim 20.

22. A hybrid vector, comprising

the DNA segment of claim 15.

23. A method of retarding, inhibiting or countering *Cryptosporidium* infection of a subject's cells comprising administering to a subject in need of such treatment an amount of the polypeptide of claim 1 capable of eliciting from the subject a cell invasion and/or development inhibitory amount of anti-*Cryptosporidium* antibodies.

24. A method of retarding, inhibiting or countering *Cryptosporidium* infection of a subject's cells comprising administering to a subject in need of such treatment an amount of an antibody capable of binding to the polypeptide of claim 1 effective to retard *Cryptosporidium* invasion of and/or development in the subject's cells.

25. A method of diagnosing *Cryptosporidium* infection,

comprising

contacting a body substance obtained from the subject with an anti-*Cryptosporidium* antibody having specificity for the polypeptide of Claim 1; and

detecting any selective binding of the antibody to any antigenic *Cryptosporidium* peptides present in the body substance.

26. A method of diagnosing *Cryptosporidium* infection, comprising

contacting a body fluid obtained from the subject with the polypeptide of claim 1; and

detecting any selective binding of the polypeptide to any anti-*Cryptosporidium* antibodies in the body fluid.

27. A kit for the diagnosis of *Cryptosporidium* infection, comprising

the polypeptide of claim 1; and

instructions for use of the kit.

28. A *Cryptosporidium* diagnostic kit, comprising

anti-*Cryptosporidium* antibodies having specificity for the polypeptide of claim 1; and

instructions for use of the kit.

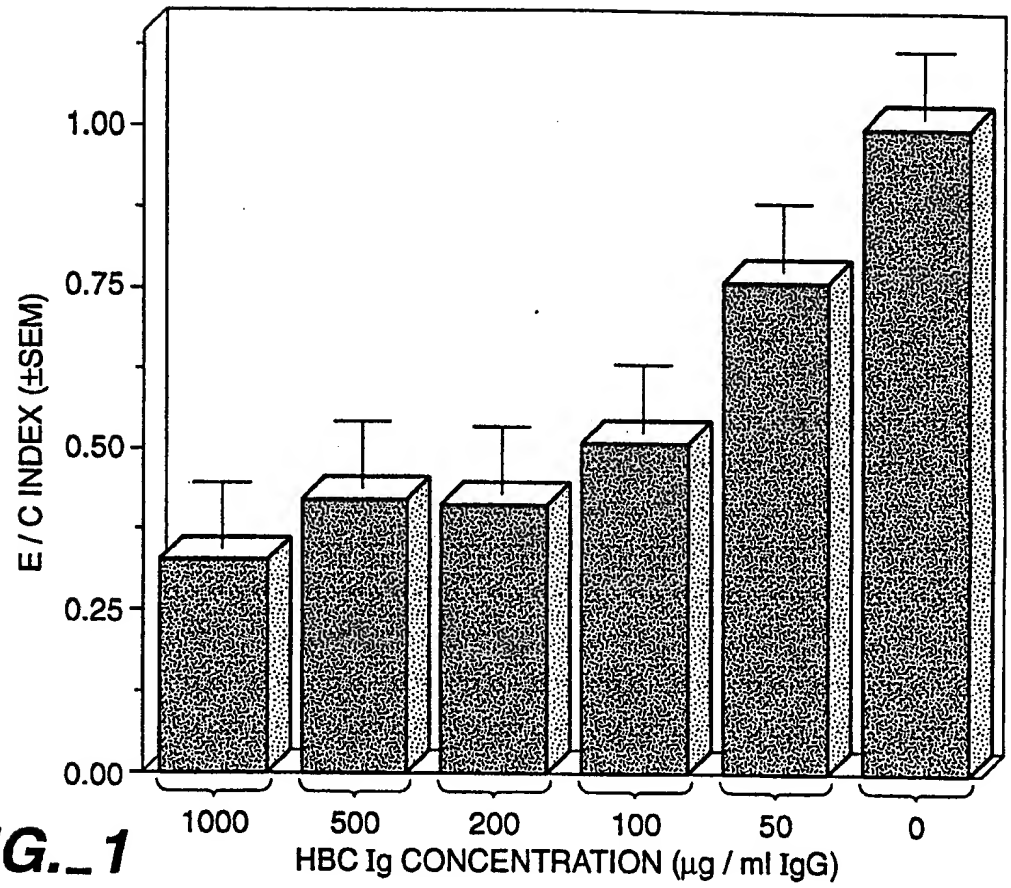
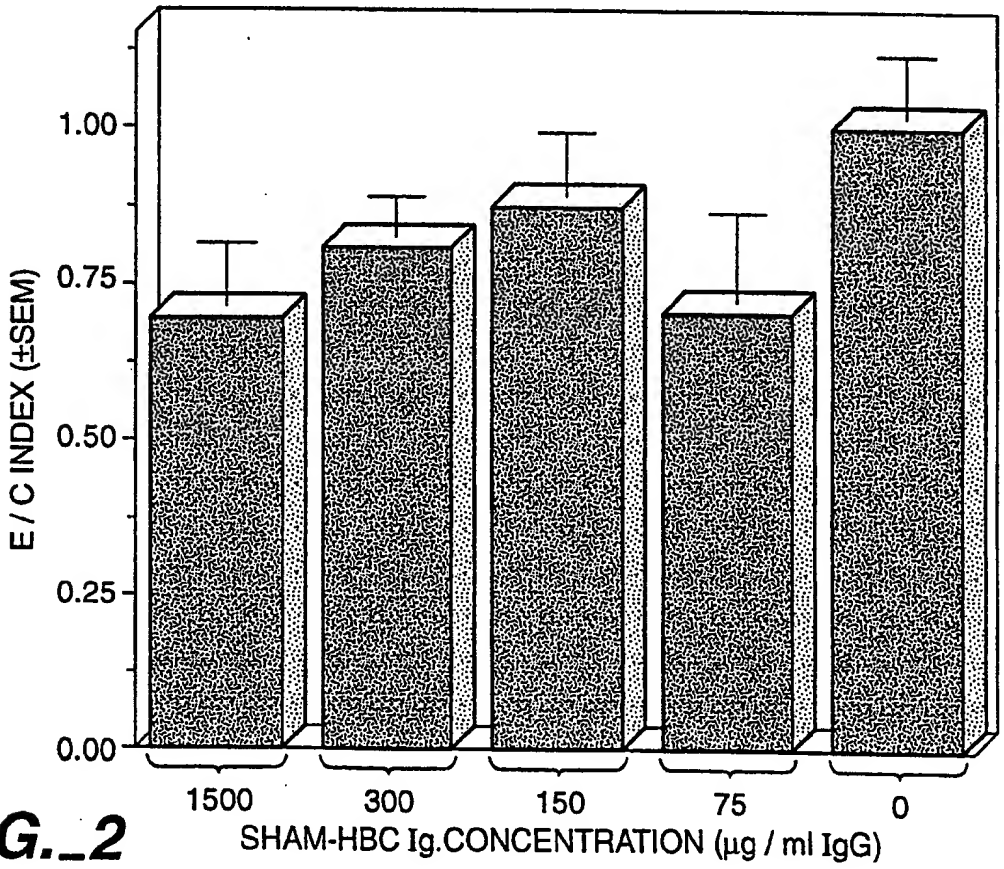

1.00
0.75
0.50
0.25
0.00
E / C INDEX (±SEM)
1000
500
200
100
50
0
HBC Ig CONCENTRATION (µg / ml IgG)

FIG._1


1.00
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0.50
0.25
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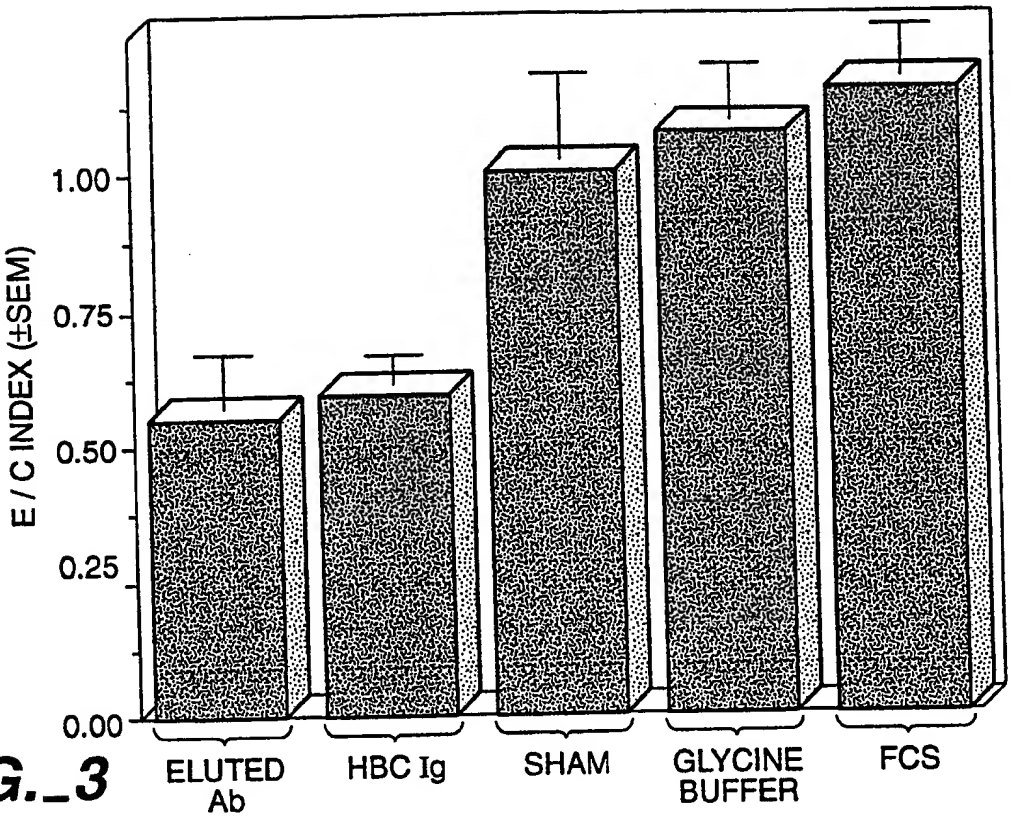
SHAM-HBC Ig.CONCENTRATION (µg / ml IgG)

FIG._2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HBC Ig
SHAM
GLYCINE BUFFER
FCS

FIG._3

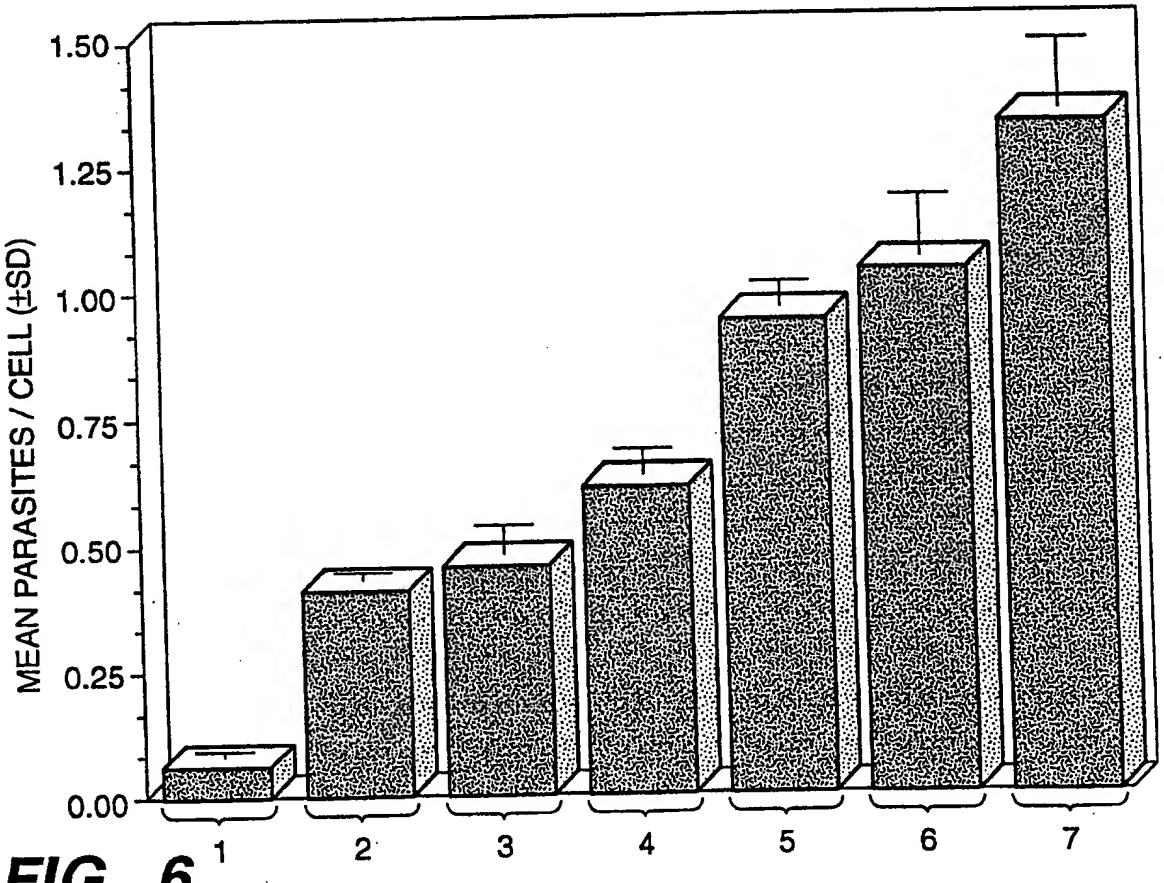
MEAN PARASITES / CELL (±SD)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1
2
3
4
5
6
7

FIG._6

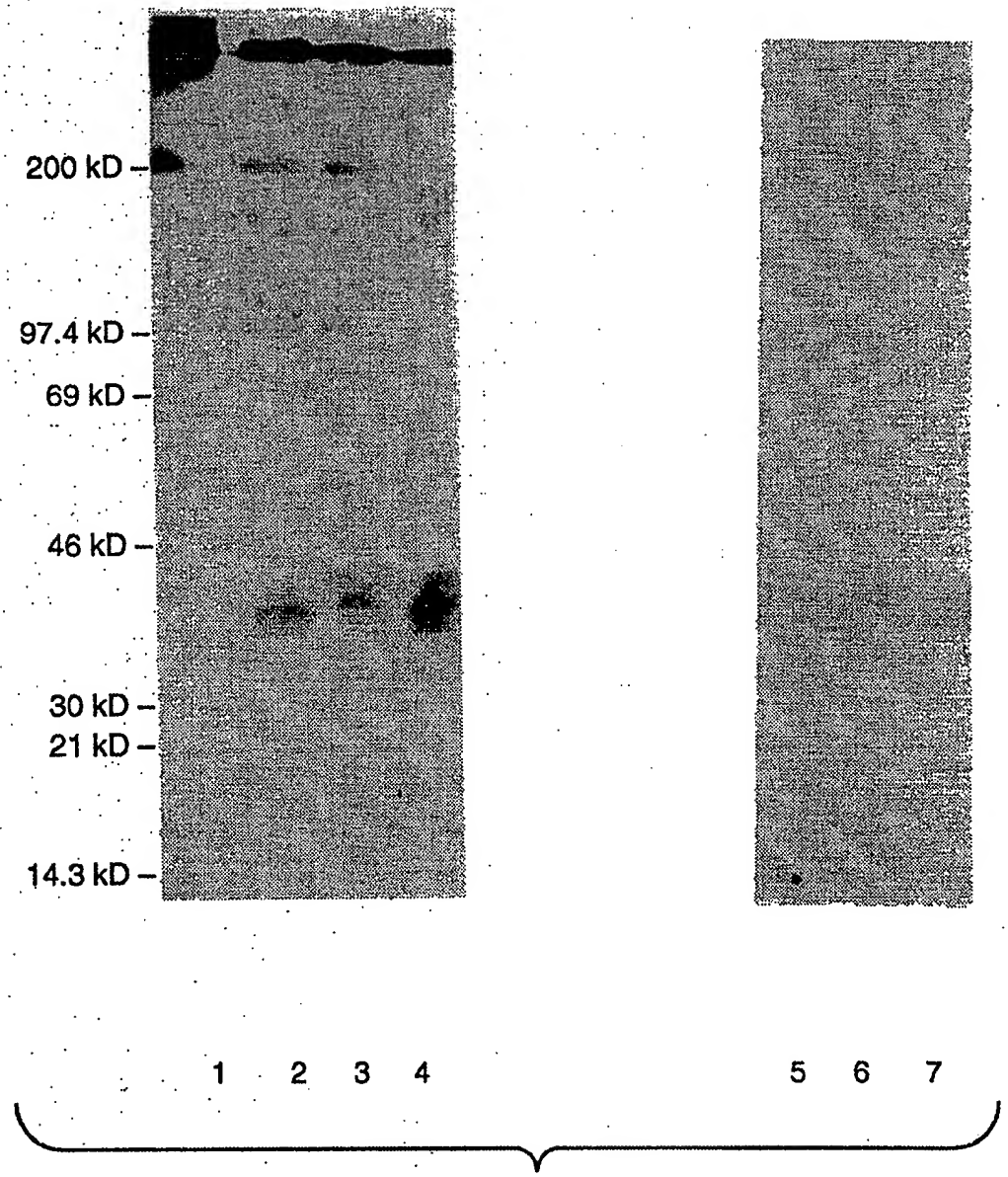

FIG._4

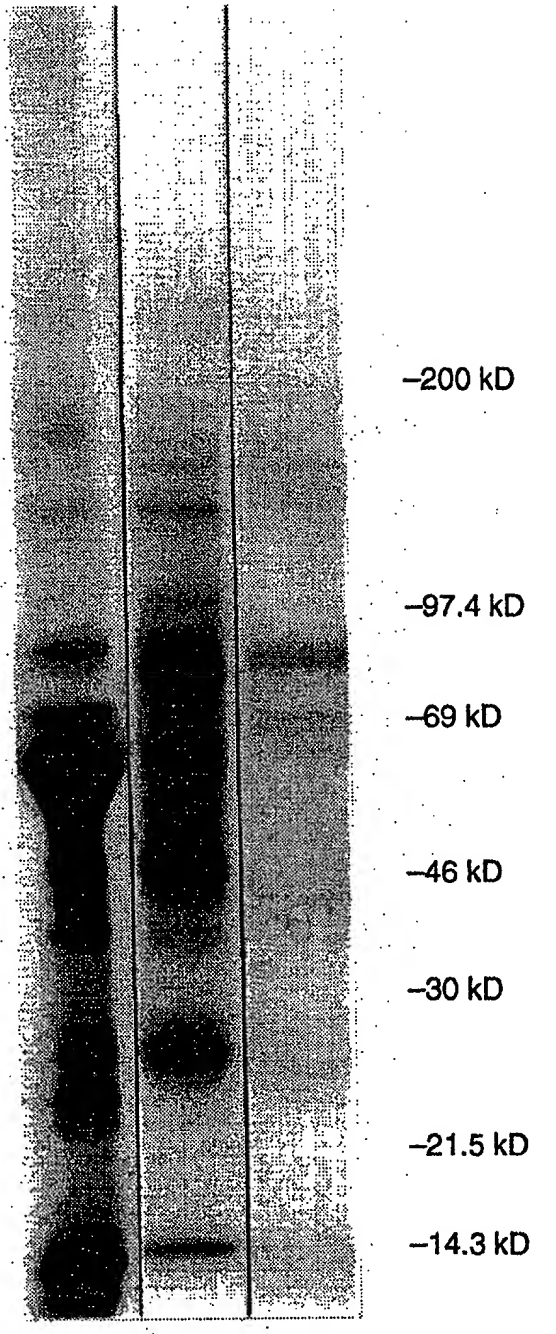

FIG._5A

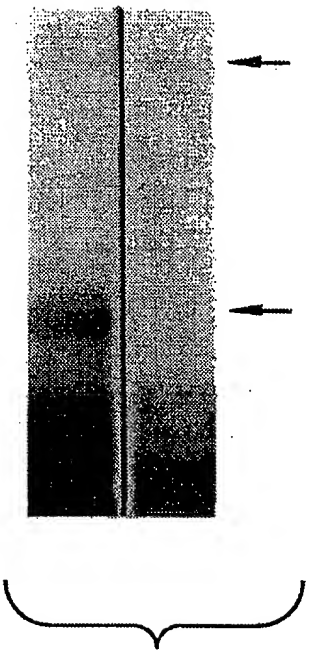

FIG._5B

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05460

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/5, 6, 7.1, 252.3, 320.1; 424/85.8, 88; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 7.1, 252.3, 320.1; 424/85.8, 88; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PIR, Swiss, GeneSeq, Medline, Aidsline, APS, WPI, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Immunology and Cell Biology, Vol.66, Part 5/6, issued October/December 1988, Lumb et al, "Electrophoretic and immunoblot analysis of *Cryptosporidium* oocysts", pp. 369-376, see entire document.	1-12, 19 23
Y	US,A, 4,784,941 (Watanabe et al) 15 November 1988, see entire document.	19
X	The Journal of Immunology, Vol.143, No.4, issued 15 August 1989, Riggs et al, "Neutralization-Sensitive Epitopes are Exposed on the Surface of Infectious *Cryptosporidium parvum* sporozoites", pp. 1340-45, see especially pages 1343-44.	23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 1993	17 SEP 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHRISTINE NUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05460

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Infection and Immunity, Vol. 59, No. 3, issued March 1991, Bjorneby et al, "Monoclonal Antibody Immunotherapy in Nude Mice Persistently Infected with *Cryptosporidium parvum*", pp. 1172-76, see entire document.	24
X	Infection and Immunity, Vol. 60, No. 4, issued April 1992, Uhl et al, "Neutralization-Sensitive Epitopes Are Conserved among Geographically Divers Isolates of *Cryptosporidium parvum*", pp. 1703-06, see entire document.	25, 28
X	Canadian Journal of Zoology, Vol. 68, issued 1990, Tilley et al, "Electrophoretic characterization of *Cryptosporidium parvum* (KSU-1 isolate) (Apicomplexa: Cryptosporidiidae)", pp. 1513-19, see especially pages 1515-18	26, 27
X	Journal of Protozoology, Vol. 38, No. 6 issued November-December 1991, Petersen et al, "Characterization of a *Cryptosporidium parvum* Sporozoite Glycoprotein", pp.20S-21S, see entire document.	13-18, 20-22
X	Journal of Protozoology, Vol. 38, No. 6 issued November-December 1991, Nelson et al, "Identification and Isolation of *Cryptosporidium parvum* Genes Encoding Microtubule and Microfilament Proteins", pp. 52S-55S, see entire document.	13-18, 20-22
X	Journal of Protozoology, Vol. 38, No. 6 issued November-December 1991, Dykstra et al, "Construction of Genomic Libraries of *Cryptosporidium parvum* and Identification of Antigen-Encoding Genes", pp. 76S-78S, see entire document.	13-18, 20-22

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/00, 1/68, 1/70; C12N 1/20, 15/00; A61K 39/00; C07K 3/12, 15/04

...RCH REPORT

International application No.
PCT/US93/05460

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)

I. Claims 1-12, 19, and 23 are drawn to a polypeptide.
II. Claims 13-18 and 20-22 are drawn to a nucleic acid, vector, and a host cell.
III. Claim 24 is a method of administering antibodies.
IV. Claims 25-28 are drawn to a method and kit for the diagnosis with antibodies.

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.